ELECTROPHORETIC PROPERTIES OF THE VIRAL CAPSID PROTEIN IN RELATION TO THE DEPENDENT TRANSMISSION PHENOMENON OF POTYVIRUSES

By

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1978

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. F.W. Zettler whose guidance, encouragement, and friendship made the fulfillment of my educational aspirations possible.

Special thanks are due to Drs. J.E. Edwardson, E. Hiebert, D.R. Pring, and D.E. Purcifull for their guidance and helpful suggestions in the preparation of this dissertation. Appreciation is extended to Mr. R.G. Christie, Mr. S. Christie, Mr. W. Crawford, Mrs. J. Hill, and Mrs. D. Miller for their generous assistance during the course of this investigation. I am also grateful to Mr. D.W. Thornbury for technical assistance with gradient gel electrophoresis techniques.

I am indebted to my family for their moral and financial support and especially to my wife for her help and companionship throughout these years.

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Abstract of Dissertation Presented to the Graduate Council
of the University of Florida in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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June 1978

Chairman: F.W. Zettler
Major Department: Plant Pathology

This study was designed to evaluate the role of the capsid protein of potyviruses in relation to the dependent transmission phenomenon.

Three isolates of dasheen mosaic virus, five isolates of bean yellow mosaic virus, one isolate of potato virus Y, and three isolates of tobacco etch virus proved aphid transmissible in this investigation. One isolate of bean yellow mosaic virus (BYMV-WISC) and one of tobacco etch virus (TEV-NAT), however, were not transmitted by the aphid *Myzus persicae* in these trials. Of seven potyviruses tested for helper activity, dependent transmission of BYMV-WISC was only demonstrated with the aid of the RC-204 isolate of BYMV. In a different test, potato virus Y (PVY) acted as a helper of TEV-NAT.

A supernatant prepared by ultracentrifugation of a freshly prepared extract obtained from PVY-infected plants was used to transmit PVY by aphids (*M. persicae*) probing through artificial membranes in mixtures of the helper and virus preparations. Electrophoretic analyses of an active helper component preparation in polyacrylamide gels (PAGE) containing sodium dodecyl sulfate (SDS) revealed the presence of at least 10 proteins

with molecular weights ranging from 11,500 to 100,000 d. These proteins were also present in control preparations obtained from noninoculated plants. Antisera prepared against coat and inclusion protein did not react with highly concentrated preparations of the helper component.

Electrophoresis of SDS-dissociated viral coat proteins of eleven potyviruses in 10% polyacrylamide gels revealed varying degrees of capsid protein heterogeneity. The ratio of the two molecular weight components observed, designated as slow and fast forms according to their electrophoretic mobility, seemed to depend upon the purification procedure. Maintaining 'Alaska' pea plants infected with the pea mosaic isolate of BYMV under adverse growing conditions did not appreciably modify the ratio of the two molecular weight components resolved by SDS-PAGE. Complete conversion of the slow into the fast form of the viral capsid protein was observed upon prolonged storage of purified preparations at 4 C or upon incubation for 30 min of purified PVY and TEV with trypsin. The conversion of electrophoretic forms significantly modified the electrostatic properties of the pea mosaic isolate of BYMV. Storing purified preparations at 4 C for varying periods of time produced the same effect.

Considerable variation in the electrophoretic properties of freshly purified potyviruses was also revealed by cellulose acetate electrophoresis in three pH-buffer systems. A direct correlation between electrophoretic mobility in a cationic system (pH 4.0) and relative aphid transmissibility was found for five isolates of BYMV and for PVY. In contrast, two of the more readily aphid-transmissible isolates of TEV showed lower electrophoretic mobilities at pH 4.0 than two isolates

showing low or no aphid transmissibility. The increased electronegativity through cellulose acetate observed for the pea mosaic isolate of BYMV upon degradation of its coat protein and the sensitivity of PVY and TEV capsid protein to degradation by trypsin suggest that the labile portion of the coat protein of potyviruses contains basic, positively charged aminoacids. The implication of these findings is discussed in relation to the specific adsorption of virus particles to aphid stylets.

INTRODUCTION

Members of the potyvirus group of plant viruses are typically transmitted by aphids in a nonpersistent manner (Fenner, 1976; Edwardson, 1974). Some potyviruses, however, can lose their aphid transmissibility after continuous maintenance in plants in the absence of their vectors (Swenson, 1957; Swenson et al., 1964). Observations, made as early as 1936 (Clinch et al.) and later in 1960 (Watson), suggested that transmission of nonaphid-transmissible potyviruses and members of other virus groups (e.g., potexviruses and tobamoviruses) with no known vectors can occasionally occur from plants also infected with certain aphid-transmissible potyviruses known today as "helpers" (Kassanis and Govier, 1971a). This phenomenon which has also been described for some semi-persistent and persistent aphid-transmitted plant viruses is referred to as "dependent transmission" (Rochow, 1977).

The International Committee on Taxonomy of Viruses (Fenner, 1976) has accepted the evidence presented by the leading workers in the field (Govier and Kassanis, 1974a, b) who indicated that a protein other than the virus coat or inclusion protein is responsible for both the dependent and independent transmission of potyviruses by aphids.

This study, which deals witn the dependent transmission phenomenon as observed in potyviruses, was designed to i) experimentally test the current evidence presented on the nature of this phenomenon; ii) determine its applicability to various potyviruses possessing differential

rates of aphid transmissibility; and iii) assess the implication of the observed capsid protein heterogeneity of potyviruses (Hiebert and McDonald, 1973; Huttinga and Mosch, 1974) in the aphid transmission phenomena of potyviruses.

LITERATURE REVIEW

The first observation of dependent transmission of a plant virus by aphids was made by Clinch and coworkers (1936). According to these authors, neigher potato virus X (PVX) nor potato aucuba mosaic (PAMV) was transmitted by the aphid Myzus persicae from infected to healthy potato plants. However, transmission of PAMV (a possible member of the potexvirus group) occurred when potato virus A (PVA), an aphid-borne potyvirus, was also present in a mixed infection with PAMV. Potato virus X was not transmitted by M. persicae from mixed infections with either PVA or PAMV.

These observations were later confirmed by Kassanis (1961) who reported that potato virus Y (PVY) could likewise assist the transmission of PAMV. Kassanis also observed that some isolates of PAMV were more readily transmitted from mixed infections with PVY than with PVA.

The number of helper viruses aiding aphid transmission of PAMV was later expanded by Kassanis and Govier (1971b) to include seven additional potyviruses: bean yellow masaic, beet mosaic, cocksfoot streak, henbane mosaic, pepper veinal mottle, potato A, and potato Y viruses. Of these, the last two and beet mosaic virus also helped transmit PVC. In these tests, PVY was the most efficient helper of both PAMV and PVC. In the same tests, PAMV was not aided by the potyviruses PVC, turnip mosaic, or lettuce mosaic virus; or by the members of other virus groups: alfalfa mosaic (monotypic), cucumber mosaic virus (cucumovirus), tobacco mosaic

virus (tobamovirus), carnation latent virus (carlavirus), or beet yellow virus (closterovirus).

In another study, Kassanis and Govier (1971a) demonstrated that PVC and PAMV were transmitted by aphids not only from plants doubly infected with a helper virus, but also from plants singly infected with either PVC or PAMV provided the aphids previously had access to a plant infected with a helper virus. No dependent transmission occurred when this sequence was reversed. These authors (1971b) were able to transmit PVC or PAMV in the same manner by aphids, after transmission of the helper virus had been prevented by irradiation of infected leaves with ultraviolet light, and thereby concluded that the helper virus itself need not be infective.

Although initial attempts to transmit potyviruses from tissue extracts had failed, Kassanis and Govier were able to transmit PVY and PAMV from extracts presented to aphids through parafilm membranes provided the aphids had first probed on a PVY-infected leaf. Three years later, Govier and Kassanis (1974a, b) described a method for preparing tissue extracts of PVY-infected plants from which aphids could acquire the virus through parafilm membranes. These extracts were prepared by homogenization of infected leaves in ammonium acetate buffer containing chelating agents. Aphids probing through parafilm membranes into freshly prepared extracts transmitted PVY to approximately 75% of the test plants.

As reported earlier for another potyvirus, turnip mosaic virus (Pirone and Megahed, 1966), Myzus persicae was unable to transmit purified PVY from artificial membranes. Purified PVY, however, was transmitted by aphids when mixed with the virus-free supernatant obtained by centrifugation of a freshly prepared extract from PVY-infected leaves at

100,000 g for 90 min. Aphids probing supernatants stored for one day at 4 C or a few hours at 20 C were not effective in transmitting the virus. Freshly prepared supernatants also helped aphids transmit purified henbane mosaic and tobacco etch viruses. Neither potato virus X nor tobacco mosaic virus was transmitted by aphids in similar tests. A concentrated supernatant obtained from TEV-infected leaves helped aphids transmit purified PVY from mixtures presented through membranes. Kassanis and Govier concluded that a component other than the virus particle is needed for virus acquisition and transmission by aphid vectors probing through artificial membranes. These authors proposed the name 'helper component' for this factor.

Govier, Kassanis, and Pirone (1977) reported the partial purification of the helper component from PVY infected tissue. The purification procedure involved concentration of the helper component with polyethyleneglycol and preservation of its activity with magnesium ($MgCl_2$). Supernatants prepared in this manner remained active for two days at 4 C or at least eight months at -15 C. Helper activity, however, was neutralized when supernatants were incubated with proteolytic enzymes or antiserum prepared to the helper component but not by antisera prepared to the virus coat or to cylindrical inclusion protein. These authors concluded that the helper component was a previously unrecognized protein coded for by the virus in infected plants. Characterization studies of this protein involving gel filtration and ultrafiltration analyses suggested that the helper component had an estimated molecular weight of 100,000-200,000 d.

Lack or loss of aphid transmissibility has been noted for several potyviruses besides PVC (Watson, 1960), including a necrosis strain of

peanut mottle virus by Paguio and Kuhn (1976), for a strain of TEV by Simons (1976), and for isolates of bean yellow mosaic virus (Swenson, 1957; Swenson et al., 1964; Evans and Zettler, 1970; Kamm, 1969). The necrosis strain of peanut mottle was transmitted by both M. persicae and Aphis craccivora from mixed infections with a different isolate of the same virus, and the TEV isolate was helped by a PVY isolate. Attempts to demonstrate dependent transmission of the vectorless strains of BYMV were unsuccessful (Evans and Zettler, 1970; Kamm, 1969). Although the loss of aphid transmissibility has been associated with continuous mechanical transfer of some of these potyviruses, Swenson et al. (1964) provided evidence that mutation of viral genomes could be responsible for the appearance of exvectorial strains.

The phenomenon of dependent transmission has also been observed in the caulimovirus group by Lung and Pirone (1973, 1974). Lack of aphid transmissibility of isolates of cauliflower mosaic virus (ClMV) could not be correlated with low virus concentration in infected plants. The normally nontransmissible isolates could be transmitted by aphids from plants also infected with a transmissible isolate, or by aphids which had previously been allowed prior access to a plant singly infected with a transmissible isolate. Purified ClMV could be transmitted from artificial membranes only by aphids that had probed leaves infected with a transmissible isolate before they were allowed to probe into the purified preparations. In the same test, aphids could not transmit purified potato virus Y, tobacco etch, or pepper veinal mottle viruses. These results indicate that while there are similarities in the dependent transmission of potyviruses and caulimoviruses, a certain degree of specificity is associated with this phenomenon.

The dependent transmission of parsnip yellow fleck virus (PYFV) by the aphid Cavariella aegopodii, from plants doubly infected with anthriscus yellow virus (AYV), constitutes the only example known for this phenomenon occurring in plant viruses having a semipersistent relationship with their aphid vector (Murant and Goold, 1968). Elnagar and Murant (1976) demonstrated that aphids already carrying AYV can acquire PYFV from leaf extracts through artificial membranes.

The dependent transmission phenomenon has also been widely documented for the following persistent groups of aphid-borne plant viruses: tobacco mottle dependent on tobacco vein distorting (Smith, 1945); various isolates of groundnut rosette virus dependent on groundnut rosette 'assistor' (Hull and Adams, 1968); carrot mottle dependent on carrot red leaf (Watson et al., 1964); tobacco yellow vein dependent on tobacco yellow vein 'assistor' (Adams and Hull, 1972); and pea enation mosaic dependent on an aphid transmissible isolate of the same virus (Tsai, 1976).

A different concept of dependent transmission of a persistent plant virus involving loss of vector specificity rather than loss of aphid transmissibility was demonstrated by Rochow (1970). The aphid Rhopalosiphum padi specifically transmits the RPV isolate of barley yellow dwarf virus (BYDV). However, this aphid species can also transmit the MAV isolate of BYDV (transmitted specifically by Macrosiphum avenae) from oat plants also infected with the RPV isolate. Since the two isolates are serologically distinct, and these viruses can be readily transmitted by aphids probing purified preparations, Rochow was able to block aphid transmission of MAV by M. avenae upon addition of MAV antiserum to a RPV-MAV mixture from doubly infected plants. From the same preparation,

R. padi acquired and transmitted both the RPV and MAV. Rochow concluded that MAV nucleic acid becomes coated with RPV coat protein during simultaneous replication of the two isolates in mixed infections. Rochow (1972) referred to this phenomenon as 'heterologous encapsidation.'

Unlike the case with non- and semi-persistent plant viruses, the demonstration of the dependent transmission phenomenon for persistent plant viruses requires that both the helper and aided viruses be present in the same plant. Aphids allowed to feed first on the helper virus source and then on a plant singly infected with the aided virus do not transmit the latter.

Tobacco mosaic virus (TMV) is one of the most infectious plant viruses for which no specific vector is recognized. Pirone and Shaw (1973) were able to transmit TMV by aphids probing through purified membranes into a mixture of TMV and poly-L-ornithine (PLO). In a later publication, Pirone and Kassanis (1975) demonstrated the transmission of two other nonaphid transmissible viruses, potato virus X and tobacco rattle virus from mixtures with PLO. Transmission of purified TMV also occurred from mixtures with poly-L-lysine or when aphids fed first on preparations of poly-L-ornithine prior to their transfer to the TMV preparations. The authors concluded that a virus-homopolymer complex is required for transmission. Purified potato virus Y, however, was not transmitted in these experiments when the virus was mixed with PLO at concentrations used to transmit TMV and PVX.

MATERIALS AND METHODS

Source of Virus Isolates

Three isolates of dasheen mosaic virus (DMV) maintained in *Philodendron selloum* C. Koch. and designated as Florida (DMV-FL), Fiji (DMV-FJ), and Egypt (DMV-E) (Zettler *et al*., 1970; Abo El-Nil *et al*., 1977); and six isolates of bean yellow mosaic virus maintained in pea (*Pisum sativum* L. 'Alaska') and designated as pea mosaic (PMV), red clover (RC-204), gladiolus C and G (GLAD-C and GLAD-G), Wisconsin (WISC), and Ohio 'severe' (OH-S) (Zettler and Abo El-Nil, 1977) were obtained from Dr. F.W. Zettler at this laboratory. Bean common mosaic virus (BCMV) was the PV25 isolate of the American Type Culture Collection, and it was maintained in bean (*Phaseolus vulgaris* L. 'Bountiful'). Blackeye cowpea mosaic virus (BlCMV) maintained in cowpea (*Vigna unguiculata* (L.) Walp. 'Knuckle Purple Hull') was obtained from Dr. J.A. Lima at this laboratory. Commelina mosaic virus (CoMV) and cucumber mosaic virus (CMV) (Morales and Zettler, 1977) originally isolated in Florida from *Commelina* spp. in Broward and Palm Beach Counties, respectively, were maintained in *Commelina diffusa* Burm. The isolate of potato virus Y (PVY) and an aphid- and non-aphid transmissible strain of tobacco etch virus (TEV-AT and TEV-NAT, respectively) investigated in a previous study (Simons, 1976) were kindly supplied by Dr. J.N. Simons. Two other TEV isolates, the ATCC PV-69 (TEV-H) and an isolate originally obtained from pepper (*Capsicum annuum* L. 'Avelar') (TEV-AV) by Dr. T.A. Zitter are

maintained at this laboratory by Drs. E. Hiebert and D.E. Purcifull, respectively. Purified tobacco mosaic virus (TMV) was obtained from Dr. E. Hiebert.

Aphid Transmission of Selected Plant Viruses

Myzus persicae (Sulzer), *Aphis craccivora* Koch, and *Pentalonia nigronervosa* Coquerel were reared on pepper (*Capsicum annuum* L. 'California Wonder'), cowpea (*Vigna unguiculata* (L.) Walp. 'Knuckle Purple Hull'), and caladium (*Caladium hortulanum* Birdsey 'Candidum'), respectively.

In vivo tests. Aphids were starved 1-2 hours prior to being transferred to test plants where they were allowed 15-60 sec acquisition probes. For sequential acquisition tests, aphids were allowed 1-5 min feeding probes on the first virus source and then 15-60 sec acquisition probes on the second virus source. All aphids were transferred to test plants for test feedings of 12-20 h before being killed with an insecticidal formulation containing malathion as the active ingredient.

In vitro tests. Membrane acquisition tests were performed as described by Govier and coworkers (1977). Aphids (*M. persicae*) were allowed to probe into preparations of purified virus (virus concentration approximately 0.5 mg/ml) containing 20% (w/v) sucrose through parafilm membranes. Following a 15 min acquisition access period, aphids were transferred in groups of ten to each test plant.

To demonstrate dependent transmission, one volume of undiluted helper component preparation was mixed with half a volume of purified virus. In some tests, one volume of purified PVY inclusions (O.D. = 0.313

at 280 nm) or 0.1 M potassium chloride (KCl) was mixed with purified PVY instead of the helper component preparation.

Purification Procedures

Bean yellow mosaic virus isolates. Systemically infected 'Alaska' pea plants (without roots), harvested 10-14 days after manual inoculation, were used for purification of the six isolates of BYMV selected in this study. The purification procedure was based on previously published procedures (Hiebert and McDonald, 1973; and Jones, 1974). One hundred grams of infected tissue were homogenized in a blender with 200 ml of a cold mixture of 0.5 M potassium phosphate buffer, pH 7.5, containing 0.5 g sodium sulfite (Na_2SO_3), 50 ml chloroform, and 50 ml carbon tetrachloride. Sodium diethyldithiocarbamate (Na-DIECA, 0.01 M) was added to the extraction buffer for purification of the OH-S isolate by BYMV. The homogenized mixture was centrifuged at 4,080 g for 5 min. The pellet was discarded and the supernatant filtered through glasswool. The virus was precipitated from this supernatant by addition of 4% (w/v) polyethylene glycol (PEG, MW 6,000). After stirring for one hour at 4 C, the virus was concentrated by centrifugation at 11,700 g for 10 min. The supernatant was discarded and the virus pellet was resuspended in 0.05 potassium phosphate buffer, pH 8.2, containing 0.1% 2-mercaptoethanol (2-ME, v/v). The virus isolates were further purified by equilibrium density gradient centrifugation (120,000 g for 17 h) in 30% (w/w) suspension of cesium chloride (CsCl) prepared in the same buffer without 2-ME.

The visible virus zone located at approximately 12 mm from the bottom of the CsCl gradient was collected after centrifugation in a dropwise

manner through a needle hole punched in the bottom of the tube. The collected volume was diluted two-fold with 0.05 M potassium phosphate buffer, pH 8.2, containing 0.1% 2-ME. The preparation was clarified by centrifugation at 12,350 g for 10 min and concentrated by ultracentrifugation at 84,500 g for 90 min. The virus pellets were resuspended overnight in 0.02 M Tris-HCl buffer, pH 8.2.

The PMV and WISC isolates of BYMV were also purified according to the extraction, clarification, and concentration methods of Jones (1974). Infected 'Alaska' pea tissue was extracted in 0.5 M potassium phosphate, pH 7.0, containing 1 M urea, 0.5% thioglycollic acid (TGA) and 0.01 M Na-DIECA. The extract was clarified with chloroform (1:1, v/v) and the virus was subsequently precipitated with 4% PEG (w/v) and 0.5 M sodium chloride (NaCl). The resuspension buffer was 0.5 M potassium phosphate buffer, pH 7.0, containing 1 M urea. The virus was further purified by equilibrium density gradient centrifugation in CsCl as described above.

Potato virus Y and tobacco etch virus isolates. The PVY isolate and four TEV isolates were propagated in tobacco *Nicotiana tabacum* L. 'Havana 425,' and the infected leaves were harvested 4-6 weeks after manual inoculation. Extraction of these viruses was performed as described above for BYMV with the exception of ethylenediamine tetraacetic acid (Na_2-EDTA, 0.01 M) which was added for all extractions from tobacco. For clarification, 8% n-butanol (v/v) was added to extracts that had previously passed through three layers of cheesecloth after homogenization. This mixture was stirred overnight at 4 C before separation by centrifugation at 11,700 g for 10 min. The pellets were discarded and the supernatants containing the virus were treated with 6% PEG and stirred for one hour at 4 C before centrifugation at 11,700 g for 10 min

to concentrate the virus. The precipitates were resuspended and further purified as described for the BYMV isolates with the exception of a clarification centrifugation (12,100 g for 10 min) given before equilibrium density gradient centrifugation in CsCl.

Potato virus Y was also purified according to the method of Govier and Kassanis (1974b). One hundred grams of infected 'Havana 425' tobacco leaves were homogenized with three times their weight of an extracting solution containing 0.1 M ammonium acetate, pH 7.0, 0.02 M Na_2-EDTA, and 0.02 M Na-DIECA. The homogenate was passed through cheesecloth and clarified by centrifugation at 8,000 g for 15 min. The supernatant was then treated with 2.5% Triton X-100 (v/v) for 20 min, and ultracentrifuged at 100,000 g for 90 min. The virus pellet was resuspended overnight in 0.1 M borate (boric acid-borax) buffer, pH 8.0, and further clarified by centrifugation at 8,000 g for 10 min. The virus was pelleted again by ultracentrifugation at 100,000 g for 90 min and the pellet resuspended in 0.01 M borate buffer, pH 8.0.

Partially purified preparations of the above viruses were sometimes obtained for gel electrophoresis. Infected tissue was homogenized and clarified as described above for each group of virus. The supernatant or aqueous phase from the clarified extracts were passed through Whatman filter paper No. 2 and the filtrates were treated with 20% PEG in 0.02 M Tris buffer, pH 8.2, using 2 ml of PEG for every 5 ml of the virus preparation (Dr. E. Hiebert, personal communication). The mixture was kept at 4 C for 30 min and the virus concentrated by centrifugation at 17,300 g for 10 min. The precipitate was resuspended in 0.02 M Tris, pH 8.2, by stirring at 4 C for 3-4 h and finally clarified by centrifugation at 12,100 g for 10 min.

Cucumber mosaic virus. An isolate of CMV originally recovered from *Commelina diffusa* was propagated in 'Havana 425' tobacco and the infected leaves were harvested three weeks after manual inoculation. For purification, 100 g of infected leaves were homogenized with a blender in a chilled mixture of 200 ml 0.5 M potassium phosphate buffer, pH 7.5, containing 0.1% TGA (v/v), 0.01 M Na_2-EDTA, and 100 ml chloroform as clarifying agent. The homogenate was centrifuged at 4,800 *g* for 5 min and the aqueous phase containing the virus was treated with 9% PEG. After stirring for 1 h at 4 C, the virus was concentrated by centrifugation at 11,700 *g* for 10 min. The pellet was resuspended in 0.05 M potassium phosphate buffer, pH 7.5, containing 0.1% TGA and 0.01 M Na_2-EDTA, and then clarified by centrifugation at 12,100 *g* for 10 min. The virus was precipitated again with 20% PEG in 0.02 M Tris buffer, pH 8.2 (2.5 ml for every 5 ml of virus preparation),and reconcentrated by centrifugation at 17,300 *g* for 10 min. The virus was resuspended in 0.005 M borate buffer, pH 9.0.

Potato virus Y inclusions. Viral inclusions were purified simultaneously with PVY according to the method of Hiebert and McDonald (1973). Following filtration through cheesecloth, the homogenate was centrifuged at 13,200 *g* and the pellet containing the inclusions was retained. The supernatant was used for virus purification as described previously. The pellet was then resuspended in 2/3 of the original extraction buffer volume without Na_2-EDTA and clarified with chloroform and carbon tetrachloride (1:1, v/v). This mixture was homogenized in a blender and centrifuged at 4,080 *g* for 5 min. The pellet was discarded and the aqueous phase was recovered and subjected again to centrifugation at 14,600 *g* for 15 min. This time the supernatant was discarded and the

pellet resuspended in 0.05 M potassium phosphate buffer, pH 8.2, containing 0.1% 2-ME, and homogenized in a Sorvall Omni-mixer for one min. The homogenate was then treated with 5% Triton X-100 (v/v) and stirred at 4 C for one h prior to centrifugation at 17,300 _g_ for 15 min. The pellet was resuspended in the same buffer and centrifuged again at 17,300 _g_ for 15 min. The resulting pellet was homogenized for 30 sec and the homogenate was layered on a sucrose step gradient made up of 10 ml of 80%, 7 ml of 60%, and 7 ml of 50% (w/v) sucrose in 0.02 M potassium phosphate buffer, pH 8.2. The preparation was then subjected to rate zonal centrifugation at 44,765 _g_ for one h. The inclusions were recovered from the top of the 80% sucrose cushion by lateral puncture with a hypodermic needle. The inclusions were diluted with three times the recovered volume in 0.02 M potassium phosphate buffer, pH 8.2, and pelleted by centrifugation at 17,300 _g_ for 15 min. The pellet containing the inclusions was resuspended in either 0.02 M Tris, pH 8.2, or deionized water.

Potato virus Y helper component. Potato virus Y infected 'Havana 425' tobacco was used as propagating material for helper component purification. The procedure was followed according to Govier and coworkers (1977). One hundred grams of PVY infected leaves collected 25-30 days after manual inoculation were infiltrated under vacuum (15 p.s.i. for 10 min) with an extracting solution of 0.1 M ammonium acetate buffer, pH 9.0, containing 0.02 M Na_2-EDTA and 0.02 M Na-DIECA. The infiltrated leaves were ground in a mortar with a volume of extracting solution equal to the original weight of tissue and the homogenate was squeezed through cheesecloth and clarified by centrifugation at 8,000 _g_ for 15 min. The resulting supernatant was collected and ultracentrifuged

at 100,000 *g* for 90 min. This second supernatant was treated with 24% PEG (w/v) in 0.1 M ammonium acetate containing 0.02 M Na_2-EDTA (pH 7.0), to give a final concentration of 6% PEG (w/v). The mixture was kept at 4 C for one h and the precipitate concentrated by centrifugation at 6,000 *g* for 5 min. The pellet was then resuspended in 0.1 M ammonium acetate containing 0.02 M magnesium chloride ($MgCl_2$), pH 7.0, and the suspension was clarified by centrifugation at 6,000 *g* for 5 min. The supernatant was treated with 24% PEG (w/v) in 0.1 M ammonium acetate containing 0.02 M $MgCl_2$ to give a final concentration of 6% PEG (w/v) at pH 7.0. The precipitate was concentrated after one h of incubation at 4 C by centrifugation at 6,000 *g* for 5 min. The resulting pellet was resuspended in 0.1 M Tris, pH 7.2, containing 0.02 M $MgCl_2$, and the solution was clarified by centrifugation at 6,000 *g* for 5 min. These preparations were frozen, thawed, and further clarified by centrifugation at 6,000 *g* for 5 min before use. The same procedure was repeated using leaves from noninoculated plants as controls.

In order to demonstrate the presence or absence of the virus in infected or noninoculated leaves, the pellet obtained by ultracentrifugation (100,000 *g*) during purification of the helper component was retained and resuspended in 0.05 M potassium phosphate buffer containing 0.01 M Na_2-EDTA and 0.1% 2-ME at pH 7.5. This suspension was clarified by centrifugation at 12,100 *g* for 10 min. The resulting supernatant was treated with 20% PEG in 0.02 M Tris, pH 8.2 (2 ml PEG/5 ml virus suspension), and incubated for 30 min at 4 C. The precipitate was reconcentrated by centrifugation at 17,300 *g* for 10 min, resuspended in 0.02 M Tris, pH 8.2, and clarified by centrifugation at 12,100 *g* for 10 min.

Spectrophotometry

The absorption spectra of purified viral, inclusion, and helper component preparations were obtained with the recorder of a Beckman model 25 spectrophotometer. The virus concentration was determined from the optical density (O.D.) at 260 nm using an extinction coefficient of 2.4 mg/ml/cm (Purcifull, 1966). Corrections for light scattering were made by measuring the absorbance at 360, 350, 340, 330, and 320 nm and plotting the logarithm of the wavelength (log O.D./100) against the logarithm of the absorbance (log 100 x O.D.) in these spectrum regions and extrapolating to 260 nm (Englander and Epstein, 1957). The light scattering value at 260 nm is then subtracted from the absorbance value of the virus preparation at the same wavelength. The 260/280 nm absorbance ratio of purified preparations was routinely determined to check the purity of the purified virus preparations.

The absorption spectrum of purified PVY inclusions was obtained as described by Hiebert _et al_. (1971). Inclusions were dissociated in an equal volume of 10% sodium dodecyl sulfate (SDS) and diluted in Tris buffer or deionized water. This mixture was boiled for 1 min and centrifuged at 3,020 _g_ for 10 min. The resulting supernatant was used for spectrophotometry.

The activity of proteolytic enzymes on purified virus preparations was followed spectrophotometrically by measuring the decrease in absorbance at 320 nm of treated purified preparations as described by Chidlow and Tremaine (1971) for cowpea chlorotic mottle virus.

Electron Microscopy

The presence, integrity, and purity of extracted or purified virus and inclusion preparations were assayed with a Philips Model 200 electron microscope. Leaf extracts and purified preparations were prepared in either 1% potassium phosphotungstate for virus particles or in 2% ammonium molybdate for viral inclusions.

Light Microscopy

Epidermal strips removed from systemically infected leaves were stained in calcomine orange and 'Luxol' brilliant green as described by Christie (1967) and examined for the presence of cylindrical inclusions.

Serology

Preparation of antiserum. Antisera to PVY, TEV-AT, and TEV-NAT were prepared by injecting New Zealand white rabbits with untreated purified virus having a high degree of capsid protein homogeneity and integrity (as determined by SDS-polyacrylamide gel electrophoresis, Hiebert and McDonald, 1973). Virus preparations were standardized to a concentration of 1 mg/ml and divided into four aliquots of 0.15 ml each which were kept frozen until use. A series of three injections were given at weekly intervals to each rabbit using the foot pad technique of immunization (Ziemiecki and Wood, 1975). Each injection consisted of 0.15 ml of the purified virus preparation emulsified with an equal volume of Freund's complete (first injection) or incomplete (subsequent injections) adjuvant. A booster injection was given 2-4 weeks after the third injection.

The rabbits were bled eight days after the third injection. Rabbits were fasted for at least four hours before 30-40 ml of blood were collected in 30 ml Corex glass tubes by nicking of the marginal ear vein with a single-edge blade (Purcifull and Batchelor, 1977). The tubes containing the blood were placed in a water bath at 37 C for 45 min to promote clotting and the antiserum was then separated from red cells by centrifugation in a Sorvall table model centrifuge at 2,000 rpm for 10 min. The serum was further clarified by centrifugation at 5,000 rpm for 10 min and frozen until needed.

Serological tests. Double immunodiffusion tests (Ouchterlony) in agar gels were performed in the following media: i) a medium containing 0.8% Noble agar (Difco), 0.25% SDS (Sigma), and 1% sodium azide (NaN_3) (Sigma) all in water (w/v) (Gooding and Bing, 1970); ii) a medium containing 0.8% Noble agar, in 0.05 M Trizma (Sigma), pH 8.0 (Shepard, 1972), 0.5% SDS, and 1% NaN_3 (Gooding and Bing, 1970); and iii) a medium containing 0.8% Noble agar, 0.2% SDS, 0.7% NaCl, and 0.1% NaN_3 (Tolin and Roane, 1975). The agar media were poured in 9 cm petri dishes and the well patterns punched with an adjustable gel cutting template (Grafar Inc., Detroit, Mich.). Wells were punched in a hexagonal arrangement with a center well spaced 4-5 mm from its edge to the edge of any of the six peripheral wells. Antigens used in these tests consisted of either fresh tissue extracts or purified preparations. Approximately 1 g of tissue was homogenized with a pestle and mortar in 1 ml of deionized water for use as antigen. For tests with purified virus, about 5-10 µl of a preparation having a concentration of 0.5-1.0 mg/ml was diluted in 95-90 µl of deionized water to use per well. Purified PVY inclusions were added at a concentration of approximately 0.3 O.D. units

at 280 nm. Purified virus preparations used in tests with proteolytic enzymes and diluted with twice their volume of a dissociation solution containing SDS (for preparation of samples for polyacrylamide gel electrophoresis) were placed at a concentration of approximately 10 μg per well.

Antiserum dilutions were made with normal serum (Purcifull and Batchelor, 1977). The reactants were pipetted into their respective wells and the plates were incubated in a moist chamber at 24 C. Reactions were observed 24 to 48 h after preparation of the plates and the precipitin lines were discerned by indirect lighting from a light box. Reactants were removed after the reactions were complete and the wells filled with 15% charcoal (Norit A) in water (w/v) to reduce pigmentation around wells and stabilize precipitin lines for photographic recording.

The following antisera: PVY-709, PVY-804, PVY-I-686, TEV-650/651, and TEV-687 from the antiserum collection maintained by Dr. D.E. Purcifull at this laboratory were used in these studies.

Degradation of Viral Coat Protein

In vivo tests. Two groups of 'Alaska' pea plants infected with the PMV isolate of BYMV were placed in growth chambers, one group at 17 C and the other at 28 C, both under a 14 h light and 10 h dark controlled cycle. Infected plants were harvested 12 days after inoculation. Two other groups of 'Alaska' pea plants were maintained in a greenhouse (24-30 C) and inoculated with PMV at two dates such that when the older group of plants was harvested seven weeks after inoculation, the second group had been infected only for two weeks. Both groups of plants were manually inoculated when plants were 10-12 days old.

The infected pea plants maintained in the growth chamber at 28 C or for seven weeks in the greenhouse were harvested after they began to show signs of physiological deterioration. After harvesting, the tissue was used for purification of PMV as described above for this isolate. The purified preparations were immediately prepared for polyacrylamide gel electrophoresis (SDS-PAGE).

In vitro tests. Purified virus preparations were assayed by SDS-PAGE in order to study the possible influence of the various purification procedures on the degradation of viral coat proteins (Hiebert and McDonald, 1973).

The effect of freezing and thawing on the heterogeneity of the viral coat protein of potyviruses was investigated with purified PMV. A virus preparation was frozen immediately after purification, thawed, and frozen and thawed again before being prepared for SDS-PAGE. Purified virus preparations were also assayed after varying periods of incubation at 4 C by SDS-PAGE.

Purified PVY and TEV-AV resuspended in 0.02 M Tris buffer, pH 8.2, were selected for studying the effect of a proteolytic enzyme on these potyviruses. Trypsin 1-300 (Nutritional Biochem. Co., Cleveland, Ohio) prepared from hog pancreas was chosen for rapid protein digestion. The enzyme was prepared in 0.001 M hydrochloric acid (HCl) to a concentration of 1 mg/ml. The virus preparations were standardized to a concentration of 1 mg/ml and 1 ml of either PVY or TEV-AV purified virus was added to a quartz cuvette for spectrophotometry. After reading the optical density of the virus preparations at 320 nm, trypsin was added to 1% the weight of the virus and the change in optical density at 320 nm after 5, 10, 15, 30, 60 min, 3 and 12 h of treatment were read off the

digital display of the spectrophotometer. Proteolytic activity was destroyed immediately after each determination by withdrawing 50 μl of the treated virus preparation from the cuvette and adding 100 μl of the SDS-dissociation solution used for preparation of virus samples for PAGE. These mixtures were boiled for 1 min and 10-20 μl (per test sample) was withdrawn for gel electrophoresis and serology.

Polyacrylamide Gel Electrophoresis

The electrophoretic analysis of viral coat and inclusion proteins in polyacrylamide gels containing SDS was performed as described by Weber and Osborn (1969) and as modified by Hiebert and McDonald (1973).

Electrophoresis was carried out in the Ortec 4010/4011 (Ortec Inc., Oak Ridge, Tenn.) vertical slab apparatus. Gel slabs 75-80 mm in height were cast to a 6 or 10% acrylamide concentration (6 or 10 ml of a mixture of 30 g acrylamide and 0.8 g N,N-methylene-bis-acrylamide, respectively) in 7.5 ml sodium phosphate buffer, pH 7.2, 0.15 ml 10% SDS, 0.045 ml N, N, N', N-tetramethylethylenediamine (TEMED), 1.2 ml ammonium persulfate (15 mg/ml), and deionized water to a total of 30 ml. The well and cap gels were prepared by mixing 1.2 ml of the sodium phosphate buffer, 7.2 ml deionized water, pH 7.2, 0.2 ml 10% SDS, 3 ml acrylamide, 0.04 ml TEMED, and polymerized with 0.3 ml ammonium persulfate. Proteins were dissociated for electrophoresis by incubation of one volume of a 1 mg/ml virus preparation in two volumes of a dissociation solution containing 0.1 ml sodium phosphate buffer, 0.25 ml 10% SDS, 0.025 ml 2-ME, and 0.25 ml 60% sucrose. Viral inclusions were dissociated in preparations having a protein concentration of approximately 3 O.D. units at 280 nm. The viruses and inclusions were boiled in the dissociation solution and

10-20 µl of the sample was layered per well. Serum albumin (67,000 d); glutamate dehydrogenase (53,000 d); carbonic anhydrase (29,000 d); and tobacco mosaic virus coat protein subunits (17,500 d), prepared to 5 mg/ml concentrations, were used as markers for molecular weight determinations. Purified preparations of the helper component were used at a concentration of about 35 O.D. units at 280 nm in order to resolve all proteins present in these preparations. Approximately 10 µl of sample were layered per well.

Electrophoresis was carried out at 160 V with the Ortec 4100 pulsed constant power supply at 300 pulses per second and 90 mA current. The migration of the proteins was followed by including bromophenol blue (0.03% in 30% sucrose, v/v) as an indicator dye. Following electrophoresis the gels were stained in a solution containing 50% methanol, 10% glacial acetic acid (v/v), and 0.1% Coomasie brilliant blue R-250 (w/v). The gels were destained in several changes of a solution of 10% methanol and 7.5% glacial acetic acid (v/v). The relative electrophoretic mobility of the proteins was determined by measuring the distance migrated in relation to the marker carbonic anhydrase.

Polyacrylamide Gel Gradient Electrophoresis

Purified preparations of the helper component, obtained from PVY infected leaves, were also analyzed in continuous-density acrylamide gradient (12-16.5%) gels.

Electrophoresis was carried out in a vertical gel slab apparatus with the Ortec Tris-sulfate-borate system (1.5 M Tris sulfate-0.065 M Tris borate). The 12.0% gel mixture was prepared by adding 2.94 ml Tris sulfate, 11.25 ml acrylamide-bis (22.0-6.0%), 0.24 ml 10% SDS,

4.45 ml deionized water, and 1.2 ml ammonium persulfate. The volumes of these reactants for the 16.5% gel were 2.94, 15.0, 0.24, 0.62, and 1.2 ml, respectively. The 12-16.5% gradient was formed with the aid of a gradient maker to a height of 14.5 cm. A stacking gel (5.5%) was prepared by mixing 1 ml 0.3 M Tris-sulfate, 2 ml acrylamide, 0.08 ml 10% SDS, 0.92 ml deionized water, 3μl TEMED and 4 ml ammonium persulfate. This gel was layered over the separation gradient gel to height of 2 cm. The well gel (8.8%) consisted of 1 ml, 0.3 M Tris-sulfate, 3.2 ml acrylamide, 0.08 ml 10% SDS, 3μl TEMED and 3.7 ml ammonium persulfate.

The samples were prepared by mixing 5-10 μl of the active helper component and control preparations with 10 μl of a dissociation solution containing 3% SDS (w/v), 3% 2-ME, and 10% glycerol (v/v) in Tris sulfate. Electrophoresis was conducted at constant voltage (80 V) with an ISCO 490 (Instrumentation Specialties Co., Lincoln, Nebraska) power supply for 19 hours. The gels were stained and destained as described previously.

Cellulose Acetate Electrophoresis

The electrophoretic behavior of the viral coat and PVY-inclusion proteins of the viruses tested in this study was carried out on Titan III cellulose acetate 77 x 26 mm plates (Helena Lab., Beaumont, Texas) using a procedure similar to that recommended for separation of serum proteins.

Three different buffer systems were used in this study. One buffer system involved the use of the Ortec Tris-sulfate-borate system (pH 9.0) which was prepared by diluting 10 ml of Tris sulfate buffer in 200 ml to soak the plates, and 1:320 Tris-borate in deionized water for the tank buffer (Dr. E. Hiebert, personal communication). Another system involved

the use of sodium phosphate buffer, similar to that used for SDS-PAGE but prepared without SDS and adjusted to pH 7.0 with HCl. Twenty milliters of this buffer were added to 200 ml of deionized water to soak plates and 32 ml into 1,280 ml of deionized water for electrophoresis. A cationic system (for potyviruses) at pH 4.0, similar to that described for separation of basic proteins (Ortec) was prepared by diluting 10 ml of a 0.48 M potassium acetate buffer (48 ml of 1.0 N KOH, and 27 ml glacial acetic acid in 100 ml of deionized water) into 200 ml deionized water to soak the cellulose acetate plates. For the tank buffer, a 0.65 M solution of Beta-alanine (29 g Beta-alanine, 34 m glacial acetic acid brought up to 500 ml with deionized water) was diluted 1:200 parts in deionized water.

The cellulose acetate plates were soaked in the buffers for 15 min prior to application of the protein samples. Untreated purified viral preparations (with a minimum concentration of 1 mg/ml for virus and 3.0 O.D. units of PVY inclusion protein at 280 nm) were applied onto the cellulose acetate strips with either a Titan serum applicator or a 5 μl pipette. Samples were applied 1.27 cm from the cathode end in the pH 9.0 system, and from the anode end in the pH 4.0 system. For the pH 7.0 system the sample was applied 2.54 cm from the cathode end. Three replicates of each sample were prepared per run, and at least two runs were carried out to determine the electrophoretic mobility of each virus.

Electrophoresis was carried out at 300, 300, and 160 V (constant voltage) for the pH 9.0, 4.0, and 7.0 systems, respectively, using a Shandon V-2541 (Shandon Scientific Co., London, England) power supply. All systems were standardized to run for approximately one hour.

The cellulose acetate plates were stained in a solution containing 0.1% Coomasie brilliant blue R-250 and 5% trichloroacetic acid (w/v) for 10-15 min. The plates were destained in three successive washes of 5% acetic acid (v/v) for 2 min each and then dehydrated for the same time in methanol. After air-drying for 5 min, the plates were placed in an oven at 100 C until completely dry.

The relative electrophoretic mobility of the proteins was determined by dividing the distance migrated by the protein front by the migration distance of bromophenol blue, for the pH 7.0 and 9.0 systems. Since this dye decomposed and did not migrate at pH 4.0, another dye, methyl green (0.1%), was used for this acidic system. This dye (Fisher Scientific Co., Fair Lawn, N.J.) was acid-resistant and migrated in the same direction as potyvirus coat protein at pH 4.0.

RESULTS

Aphid Transmission of Selected Plant Viruses

Dasheen mosaic virus isolates. Dasheen mosaic virus (DMV) was included in this study due to the comparatively low aphid transmissibility of the Florida isolate (DMV-FL) and the previous failure to transmit the Fiji isolate (DMV-FJ) by means of aphids. No vector data were available for the Egyptian isolate of this virus (DMV-E) (F.W. Zettler, personal communication).

The results from this test have been published elsewhere (Morales and Zettler, 1978) and are summarized in Tables I and II. None of the three DMV isolates was transmitted to more than 10% of the test plants by single individuals of *M. persicae*. Similarly, in a test involving the DMV-FL isolate, *A. craccivora* did not transmit the virus to more than 10% of the test plants. *Pentalonia nigronervosa*, a common pest of certain aroids, did not transmit DMV-FL (or any of the other isolates) even when 20 aphids were used per test plant. In this study, aphid transmission rate of DMV was considered low when compared with the results obtained in a parallel test where a single individual of *M. persicae* used per plant was able to transmit blackeye cowpea mosaic virus (BlCMV) from and to 'Knuckle Purple Hull' cowpea resulting in infection of 53.5% of the test plants (Table I). The high aphid transmissibility of BlCMV had already been demonstrated by Zettler *et al*. (1967). *Myzus persicae*, however, transmitted DMV-FL, DMV-FJ, DMV-E, to 50, 40, and 45%,

Table I. Comparative aphid transmissibility of three dasheen mosaic virus isolates by *Myzus persicae*.

No. Aphids per Plant	Trial No.	Virus Isolate			
		DMV-FL	DMV-FJ	DMV-E	BlCMV[a]
1	1	0/10[b]	1/10	0/10	7/15
	2	1/10	1/10	0/10	9/15
2	1	1/10	2/10	2/10	
	2	3/10	3/10	2/10	
6	1	4/10	3/10	3/10	
	2	6/10	5/10	6/10	

[a]Blackeye cowpea mosaic virus (BlCMV) transmitted from and to cowpea *Vigna unguiculata* (L.) Walp. 'Knuckle Purple Hull' was included as control.

[b]No. of *Philodendron selloum* infected over number of plants inoculated.

Table II. Comparative transmissibility of the Florida isolate of dasheen mosaic virus by three aphid species.

Trial No.	Aphid Species		
	Myzus persicae	*Aphis craccivora*	*Pentalonia nigronervosa*
1	6/10[a]	3/10	0/10
2	6/10	3/10	0/10
3	7/10	3/10	0/10
4	6/10	4/10	0/10

[a]Number of *Philodendron selloum* infected over total number of plants inoculated by placing six aphids per plant.

respectively, of the Philodendron selloum test plants when six aphids were placed per plant. The results from this test also demonstrated that DMV-FJ was aphid transmissible by M. persicae with the same efficiency of the other two isolates (factorial analysis). Myzus persicae proved to be a significantly better (P = 0.01) vector of DMV-FL than A. craccivora. Transmission rates of 63 and 33% were recorded for each species, respectively.

In order to test the possibility of increasing the efficiency of aphid transmission of DMV-FL, individuals of M. persicae were allowed to feed on either 'Knuckle Purple Hull' cowpea infected with BlCMV, a virus serologically related to DMV (Lima et al., 1976), or C. diffusa plants infected with commelina mosaic virus (CoMV), a virus with a high rate of transmission (up to 70% transmission by two individuals of M. persicae from and to C. diffusa, Morales and Zettler, 1977). After 5-10 min access periods, the aphids were transferred to DMV-FL infected P. selloum for acquisition probes of 15-60 sec. The aphids were then transferred singly to healthy P. selloum seedlings for transmission probes. In these tests, M. persicae did not transmit DMV-FL to test plants (0/5, 0/5, 0/5) after first probing BlCMV-infected plants; and only one test plant (0/5, 1/5, 0/5) was infected when aphids first fed on C. diffusa infected with CoMV. Myzus persicae transmitted CoMV in the same test to 60% of inoculated C. diffusa plants (12/20) when placed singly on each plant after 15-60 acquisition probes on CoMV-infected C. diffusa. In a parallel test, M. persicae transmitted DMV-FL to 9/10 P. selloum plants when aphids were allowed 15-60 sec acquisition probes in infected P. selloum and then transferred in groups of 20 to each test plant.

Bean yellow mosaic virus isolates. Transmission rates by single individuals of *M. persicae* for the PMV, RC-204, GLAD-C, GLAD-G, WISC, and OH-S isolates of BYMV from and to 'Alaska' pea were 23, 40, 20, 20, 0, and 53%, respectively. Transmission rates for the RC-204, WISC, and OH-S isolates from 'Alaska' pea to 'Bountiful' bean were 65, 0, and 70%, respectively, using three aphids per plant. It was apparent from these studies that the WISC isolate was not transmitted in these tests by *M. persicae* to either 'Alaska' pea or 'Bountiful' bean (Table III).

Seven potyviruses were consequently tested for helper activity with the WISC isolate of BYMV. Since the PMV and GLAD-C isolates rarely infect 'Bountiful' bean systemically, and the RC-204 isolate induces a mosaic unlike the severe mosaic, stunting, and epinasty characteristic of the WISC isolate when manually inoculated in 'Bountiful' bean (Zettler and Abo El-Nil, 1977), these aphid-transmissible BYMV isolates were also included in this test. Blackeye cowpea mosaic virus rarely infects bean systemically, and bean common mosaic virus induces distinctive mosaic symptoms in this host. The results from this test (Table IV) indicated that only the RC-204 isolate of BYMV was an inefficient helper of the WISC isolate in tests involving singly and doubly infected plants (Table IV). Attempts to transmit the WISC isolate with *M. persicae* from the infected test plants to 30 'Bountiful' bean plants (2 aphids/plant) proved unsuccessful.

Potato virus Y and tobacco etch virus isolates. Attempts were made to reproduce the work of Simons (1976) with PVY and two TEV isolates (TEV-AT and TEV-NAT) supplied by the author for this study.

The three viruses were maintained in pepper (*Capsicum annuum* L. 'California Wonder') (CW), and *M. persicae* (2 aphids/plant) was used to

Table III. Comparative aphid and mechanical transmissibility of six bean yellow mosaic virus isolates.

Isolate	Trial No.	Test Plant			
		'Alaska' pea		'Bountiful' bean	
		M. persicae [a]	Manual [b]	_Myzus persicae_	Manual
PMV	1	2/10 [c]	+		- [d]
	2	5/20			
RC-204	1	4/10	+	6/10	+
	2	8/20		7/10	
GLAD-C	1	2/10	+		-
	2	4/20			
GALD-G	1	2/10	+		-
	2	4/20			
WISC	1	0/20	+	0/10	+
	2	0/40		0/20	
OH-S	1	5/10	+	8/10	+
	2	11/20		6/10	

[a] One aphid per 'Alaska' pea plant and three aphids per 'Bountiful' bean plant.

[b] Thirty plants of each species inoculated per isolate.

[c] Number of plants infected over total number of plants inoculated.

[d] - = no systemic infection.

Table IV. Dependent transmission trials with the Wisconsin isolate of bean yellow mosaic virus and seven other potyviruses tested for helper activity.

Virus Tested as Helper	Host plant of Virus Tested as Helper	Dependent Transmission of BYMV-WISC[a] Trial No.	
		1	2
PMV	'Alaska' pea	0/30[b]	0/30
RC-204	'Alaska' pea	1/30	2/30
GLAD-C	'Alaska' pea	0/30	0/30
BlCMV	'Knuckle Purple Hull' cowpea	0/30	0/30
BCMV	'Bountiful' bean	0/30	0/30
PVY	'Havana 425' tobacco	0/30	0/30
CoMV	*Commelina diffusa*	0/30	-
RC-204 + WISC[c]	'Alaska' pea	2/20	2/20

[a]Maintained in 'Alaska' pea.

[b]Number of 'Bountiful' bean plants infected with the WISC isolate of BYMV over total number inoculated with the aphid *Myzus persicae* (2 aphids/plant).

[c]Virus source plant doubly infected with the RC-204 and WISC isolates of BYMV.

transmit the viruses to a PVY-immune Italian El pepper cultivar (IE) also supplied by Dr. J.N. Simons.

Results from these tests (Table V) did not yield the high rates of transmission demonstrated by Simons for TEV-AT (96% versus 10% in this study). The latter virus did not prove transmissible from singly infected plants in 2 trials involving 30 Italian El test plants and 2 aphids per plant. This virus was transmitted, however, to 6 of 60 test plants when aphids were allowed previous acquisition probes in PVY-infected plants. The helper activity of PVY was demonstrated from either pepper or tobacco (Table V). Two other isolates of TEV (TEV-H and TEV-AV) were also transmitted by _M. persicae_ in these tests, both from 'California Wonder' pepper and/or 'Havana 425' tobacco (TEV-AV tested only from CW).

Aphid transmission of purified potato virus Y. Freshly purified PVY resuspended in 0.02 M Tris buffer, pH 8.2, was not transmitted by _M. persicae_ probing through artificial membranes even when the ionic strength of the virus preparation was adjusted with 0.1 M KCl (Table VI). Similarly, purified PVY inclusions mixed with purified PVY did not aid aphid transmission of the virus. Purified preparations of PVY used in these tests always proved infectious when manually inoculated on 'Havana 425' tobacco. In contrast, transmission of purified cucumber mosaic virus (CMV) was achieved in simultaneous tests with _M. persicae_ after acquisition of the virus from parafilm membranes in agreement with the observations of Pirone and Megahed (1966).

In these trials, transmission of purified PVY was obtained when mixed with a freshly prepared helper component preparation as reported previously by Govier and Kassanis (1974a, b). Experiments 3 and 4 were conducted with the same preparation kept frozen in aliquots.

Table V. Independent and dependent aphid transmission trials with potato virus Y and four tobacco etch virus isolates.

Virus	Host	Dependent Virus	Host	No. Plants Infected Trial No.		Test Plant[a]	Serology[b] PVY As
				1	2		
PVY	CW	-[c]	-	0/15[d]	0/15	IE	-
PVY	CW	-	-	5/15	6/15	CW	-
TEV-AT	CW	-	-	0/30	2/20	IE	-
TEV-AT	CW	-	-	0/6	1/6	H-425	-
TEV-NAT	CW	-	-	0/15	0/15	IE	-
PVY	CW	TEV-NAT	CW	0/15	2/15	IE	0/2
PVY	H-425	TEV-NAT	H-425	2/5	3/5	H-425	1/5
TEV-H	H-425	-	-	3/6	-	H-425	-
TEV-H	CW	-	-	3/6	-	IE	-
TEV-AV	H-425	-	-	5/6	-	H-425	-

[a] IE = PVY-immune Italian El pepper cvar.; CW = 'California Wonder' pepper; H-425 = 'Havana 425' tobacco.

[b] PVY antiserum used does not react with TEV.

[c] Not tested.

[d] Number of plants infected over total number inoculated.

Table VI. Aphid transmission of purified potato virus Y acquired through artificial membranes.

Preparation	Experiment			
	1	2	3	4
Purified PVY	0/4[a]	0/4	0/4	0/4
Purified PVY + helper component preparation	0/4	0/4	1/4	2/4
Purified PVY + control preparation	0/3	0/3	0/3	0/3
Purified PVY + purified PVY inclusions	-	0/4	0/4	0/4
Purified PVY + 0.1 M potassium chloride (KCl)	-	-	0/4	0/4
Purified cucumber mosaic virus (CMV)	2/4	1/4	-	-

[a]Number of 'Havana 425' tobacco test plants infected by placing 10 *Myzus persicae* individuals per plant after being permitted acquisition probes into test preparations through artificial membranes.

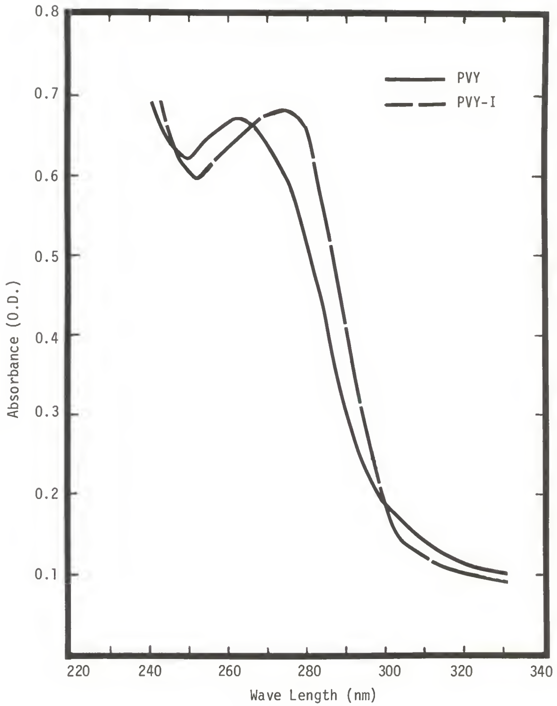
Virus and Viral Inclusion Purification

Bean yellow mosaic virus isolates. Yields of purified BYMV isolates ranged from 10 to 28 mg per kg of infected 'Alaska' pea tissue (values corrected for light scattering). The highest yield was observed for the WISC isolate (28 mg/kg tissue).

Addition of Na-DIECA to the extraction buffer was found to be necessary only for recovery of the OH-S isolate. Addition of urea was not indispensible for purification of any of the isolates. Furthermore, it was determined that buffers containing Na-DIECA must be used immediately after preparation or severe losses in virus yield occurred. Purified preparations had absorbance (A) 260/280 ratios of 1.18 and 1.2 units in agreement with values reported for this virus (Jones, 1974).

Potato virus Y and tobacco etch virus isolates. Corrected yields for PVY ranged from 10 to 20 mg of virus per kg of infected 'Havana 425' tobacco. Addition of Na_2-EDTA to the extraction buffer seemed to prevent virus losses due to aggregation as determined by electron microscopy. Clarification by centrifugation of virus preparations before equilibrium density gradient centrifugation in CsCl greatly facilitated observation and recovery of virus zones. Preparations of purified PVY exhibited typical nucleoprotein absorption spectra (Fig. 1) and had A260/280 ratios between 1.18 and 1.2. Recovery of 20 to 30 mg of PVY per kg of infected 'Havana 425' tobacco tissue was obtained using the procedure described by Govier and Kassanis (1974b). These preparations, however, contained visible amounts of contaminants as judged by their green color and A260/280 ratios of 1.4-1.6.

Figure 1. Ultraviolet absorption spectra of purified preparations of potato Y virus (PVY) and inclusions (PVY-I) in 0.02 M Tris buffer, pH 8.2. The PVY-I preparation contains 1% SDS.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance (O.D.)
PVY
PVY-I
220
240
260
280
300
320
340
Wave Length (nm)

Corrected yields for the purified TEV isolates ranged from 18.5 to 30.0 mg of virus per kg of infected 'Havana 425' tobacco tissue. Clarification of preparations before equilibrium density gradient centrifugation also resulted in improved recovery of virus zones from the CsCl gradients. Absorbance 260/280 nm ratios of 1.17-1.21, similar to those reported for this virus by Shepherd and Purcifull (1971), were obtained for these isolates.

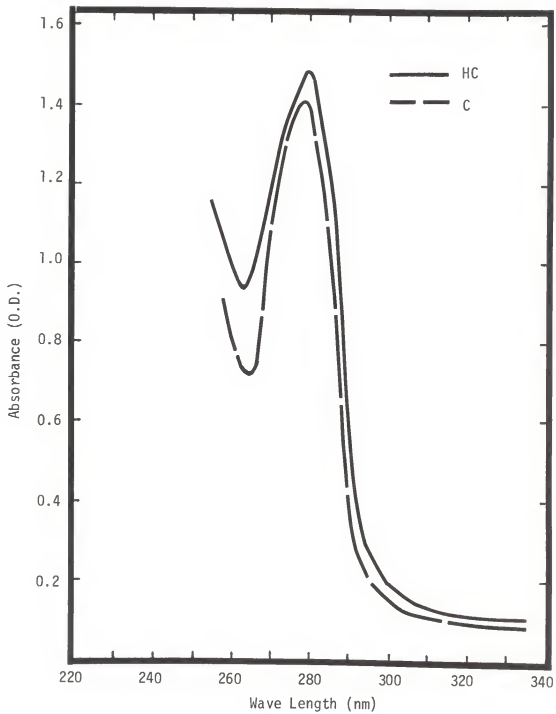
Purification of PVY and TEV isolates by the double PEG concentration method yielded in many cases colorless preparations with A260/280 ratios close to 1.2.

Cucumber mosaic virus. Corrected yields for the CMV isolate used in this study were estimated at 60 mg per kg of infected 'Havana 425' tobacco tissue. These preparations had an A260/280 ratio of 1.65 as expected for this virus (Gibbs and Harrison, 1970).

Potato virus Y inclusions. Preparations of PVY inclusions exhibited absorption spectra typical of proteins (Fig. 1) and were spectrophotometrically determined to have concentrations of 3.0-5.0 A280 units/ml/ 100 g of infected tissue. Electron microscopic examinations of these purified preparations revealed the characteristic striations of PVY inclusions demonstrated by Hiebert et al. (1971).

Purification of helper component. An active preparation of helper component and its control were nearly colorless after the final clarification step. Twenty-fold dilutions of this preparation exhibited a typical protein spectrum (Fig. 2). No apparent qualitative differences were noted between the ultraviolet spectra of the helper component and control preparations.

Figure 2. Ultraviolet absorption spectra of a purified PVY helper component (HC) preparation and control (C) obtained from noninoculated plants in 0.1 M Tris buffer containing 0.02 M $MgCl_2$, pH 7.2.


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance (O.D.)
HC
C
220
240
260
280
300
320
340
Wave Length (nm)

Potato virus Y was consistently recovered from the high speed (100,000 _g_) pellet discarded during purification of the helper component. No virus was recovered from several control preparations obtained from noninoculated plants, however.

Serology

In agreement with the report by Govier _et al_. (1977) neither the PVY coat protein nor the inclusion protein antisera used in this study reacted with highly concentrated preparations of active helper component in agar gel double immunodiffusion tests although they readily reacted with their homologous antigens. Similar results were obtained when PVY coat or inclusion protein antisera were diluted 1/2, 1/4, 1/8, and 1/16 in normal serum and tested against the helper component preparation, or when 1/20, 1/5, and 1/2 dilutions of the helper component preparation in deionized water were tested against PVY antisera.

Reciprocal double immunodiffusion tests with the TEV-AT and TEV-NAT isolates using antisera obtained to virus coat protein (predominantly in the undegraded or slow form)as determined by SDS-PAGE; Hiebert and McDonald (1973) did not reveal any serological differences between these two isolates (Fig. 3). Tests with these and two other TEV antisera obtained for this study also gave serological reactions of identity between TEV-NAT and the three aphid-transmissible isolates (TEV-AT, TEV-H, and TEV-AV) investigated here.

Figure 3. Reciprocal double immunodiffusion test with an aphid (AT) and a nonaphid-transmissible isolate of tobacco etch virus (TEV) in a medium containing 0.8% Noble agar, 1% NaN_3, and 0.5% SDS prepared in water. Center wells contain: (A) TEV-AT antiserum, (N) TEV-NAT antiserum, (ns) normal serum. Peripheral wells contain: (at) TEV-AT in sap extract from infected 'Havana 425' tobacco, (nat) TEV-NAT in sap extract from infected 'Havana 425' tobacco, (h) sap from noninoculated 'Havana 425' tobacco.

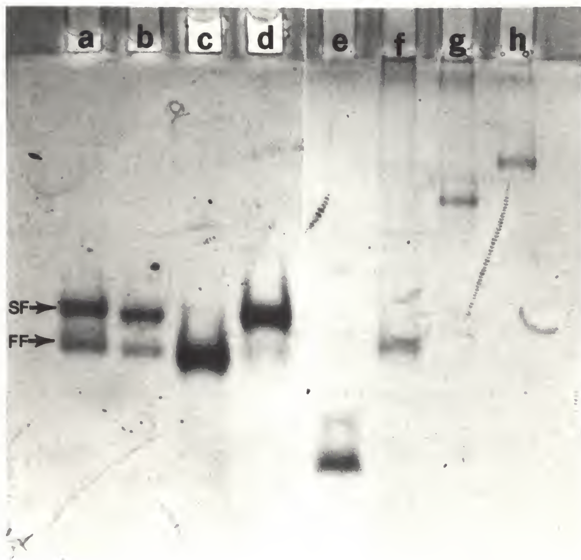
at
nat
h
at
h
nat
h
A
N
at
nat
at
at
nat
nat
at
at
h
nat
ns
at
at
nat

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of the SDS-dissociated viral coat protein of selected potyviruses. When the SDS-dissociated coat protein of the PMV, RC-204, GLAD-C, and WISC isolates of BYMV (simultaneously purified prior to electrophoresis) were assayed by SDS-PAGE in a 10% gel, considerable variation was observed in the ratios of the two molecular weight components resolved (Fig. 4). These components,which have been referred to as slow and fast forms according to their electrophoretic mobility (Hiebert and McDonald, 1973; Huttinga and Mosch, 1974),were observed for the PMV and WISC isolates whereas only the fast form of the RC-204 or the slow form of the GLAD-C isolate was present.

Since Hiebert and McDonald (1976) demonstrated that the condition of the coat protein might have a marked effect on the physical and serological properties of potyviruses, it was imperative to obtain viral coat protein with adequate capsid protein homogeneity. The conversion of the slow into the fast form has been observed to occur during storage of purified preparations (Hiebert and McDonald, 1973) and upon incubation of potyviruses in solutions containing proteolytic activity (Huttinga and Mosch, 1974). Hiebert and McDonald (1973, 1976) suggested that the ratio of the two components seems to depend on the purification procedure, while some degradation could take place in situ. Based on this hypothesis, the effect of adverse growth conditions was investigated with PMV-infected 'Alaska' pea maintained in growth chambers at 17 and 28 C, or in a greenhouse for 2 or 7 weeks. After the tissue was harvested and the virus from each treatment purified, their coat proteins were immediately prepared for SDS-PAGE.

Figure 4. Electrophoretic forms of the SDS-dissociated capsid protein subunit of four bean yellow mosaic virus isolates and marker proteins in a 10% polyacrylamide gel. Samples from left to right are (a) PMV, (b) WISC, (c) RC-204, (d) GLAD-C, (e) TMV, MW 17,500 d, (f) carbonic anhydrase, MW 29,000 d, (g) glutamate dehydrogenase, MW 53,000 d, (h) bovine serum albumin, MW 67,000 d. Arrows show (SF) slow form, (FF) fast form.


a
b
c
d
e
f
g
h
SF
FF

Results from these tests (Fig. 5) indicated that neither the temperature nor the senescence process selected significantly modified the ratio or position of the molecular weight components observed. Furthermore, the capsid protein of PMV exhibited considerably more homogeneity in these experiments, being predominantly in the slow or undegraded form. Freezing and thawing of a purified preparation of PMV did not alter the ratio of the two components (Fig. 5).

In all subsequent trials, all of the BYMV isolates studied were obtained with their coat protein subunits in the slow or undegraded form. Conversion of these predominantly slow forms into the fast form occurred for all BYMV isolates except OH-S upon storage of purified preparations at 4 C for 2-3 months (Fig. 6). Molecular weight estimates for the components resolved (Table VII) indicated that the conversion of the slow into the fast form seems to result from the loss of a polypeptide fragment with molecular weight ranging from 3,500 to 5,000 d.

Generally, only the heavier molecular weight component corresponding to the slow form of the capsid protein subunit was observed in preparations of purified PVY and TEV isolates. Increasing amounts of the faster moving component, however, were observed when PVY was purified according to the method of Govier and Kassanis (1974b), or when the virus was recovered from the high speed (1000,000) pellet obtained during purification of the helper component (Fig. 7). Conversion of the slow to the fast form upon storage appears to involve the loss of a potypeptide fraction of about 5,000 d for the TEV isolates and 6,700 d for PVY. These potyviruses, however, proved far more refractory to degradation upon storage at 4 C for periods of up to six months than the BYMV isolates. A purified PVY preparation maintained for over three years at

Figure 5. Electrophoresis of the SDS-dissociated capsid protein subunit of the pea mosaic isolate of bean yellow mosaic virus: A) purified 2 weeks after inoculation (a), purified 7 weeks after inoculation (b); B) maintained in a growth chamber at 17 C (a); maintained in a growth chamber at 28 C (b) and then purified; C) freshly purified virus (a), incubated for 6 months at 4 C after purification (b), purified virus frozen and thawed twice after purification (c), in 10% polyacrylamide gels.

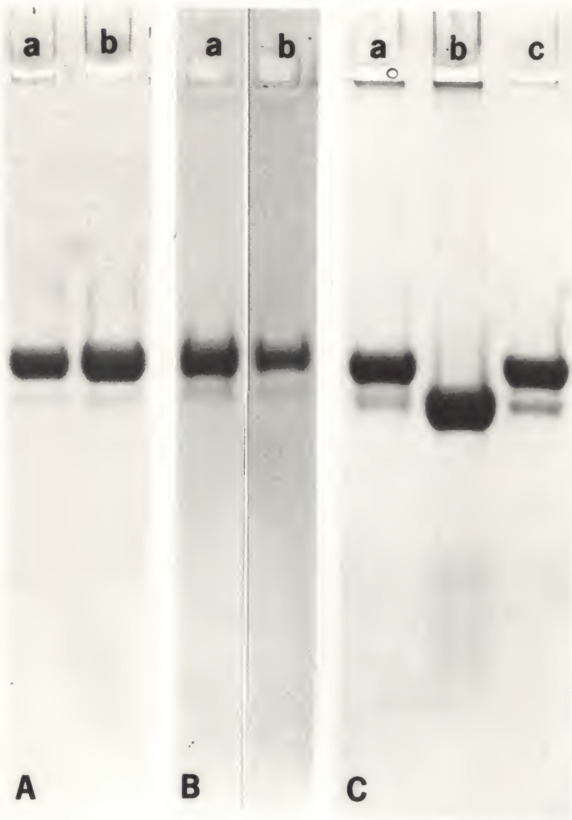

a
b
a
b
a
b
c
A
B
C

Figure 6. Electrophoresis of the SDS-dissociated capsid protein subunits of freshly purified and stored preparations of five bean yellow mosaic virus isolates in 10% polyacrylamide gels containing SDS. Samples from left to right are (a) fresh PMV, (b) stored PMV, (c) fresh WISC, (d) stored WISC, (e) fresh RC-204, (f) stored RC-204, (g) fresh OH-S, (h) stored OH-S, (i) fresh GLAD-G, (j) stored GLAD-G.

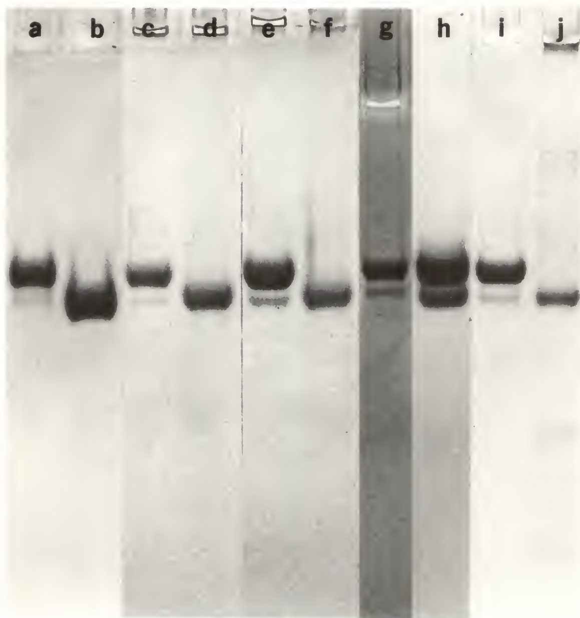

a
b
c
d
e
f
g
h
i
j

Table VII. Molecular weight estimates of the SDS-dissociated coat protein subunits of eleven potyviruses analyzed by polyacrylamide gel electrophoresis.

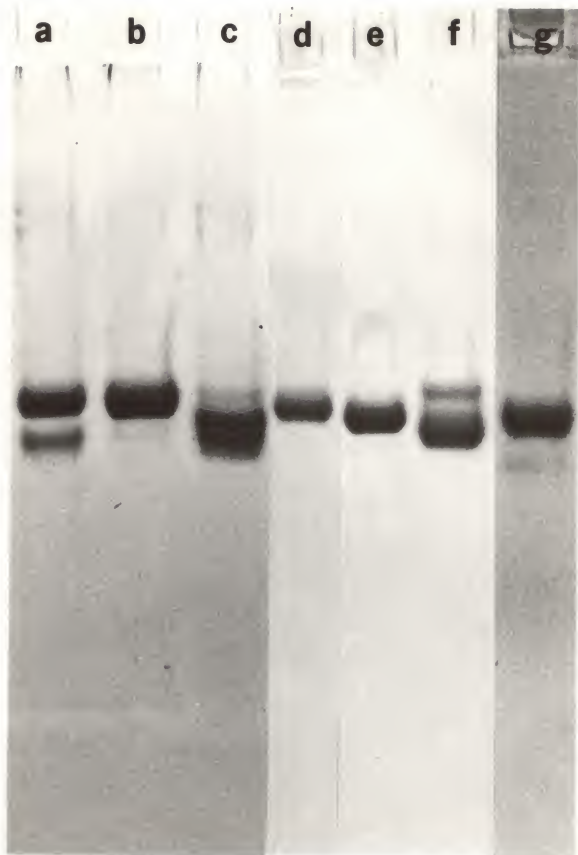
Virus	Molecular Weight[a]	
	Slow Form	Fast Form
PMV	33.5-34.0[b]	30.0-28.0
RC-204	33.0-32.0	29.0-27.5
GLAD-C	33.0-32.5	28.0-27.5
GLAD-G	33.6-33.0	29.0-28.5
WISC	32.5-31.0	28.5-27.0
OH-S	31.6-31.4	28.5-27.5
TEV-AT	31.0-30.5	-[c]
TEV-NAT	31.0-30.0	-
TEV-H	31.5-30.0	26.0-25.5
TEV-AV	32.0-31.5	27.5-26.0
PVY	34.0-33.7	28.0-27.0

[a]Molecular weights determined in 10% gels with the exception of TEV-AV (6%). Estimates are expressed as MW x 10^3 daltons.

[b]Molecular weight ranges represent the higher and lower estimates obtained in at least three separate determinations.

[c]Not tested.

Figure 7. Electrophoresis of the SDS-dissociated capsid protein subunits of freshly purified and stored preparations of potato virus Y (PVY) and three isolates of tobacco etch virus (TEV) in 10% polyacrylamide gels containing SDS. Samples from left to right are (a) PVY purified by differential centrifugation according to the method of Govier and Kassanis (1974b), (b) PVY purified by equilibrium density gradient centrifugation, (c) PVY preparation maintained for over three years at 4 C after purification, (d) TEV-AT isolate, fresh; (e) TEV-NAT isolate, fresh; (f) purified PVY preparation kept at 4 C for a year, (g) freshly purified TEV-H isolate.


a
b
c
d
e
f
g

4 C still contained some coat protein in the slow form when assayed by SDS-PAGE (Fig. 7c).

Effect of trypsin on the capsid protein of potato virus Y and tobacco etch virus. Incubation of purified PVY and TEV-AV with trypsin, resulted in the rapid conversion (Table VIII) of their respective heavier molecular weight forms to a faster migrating form corresponding to the fast form obtained upon storage of these viruses at 4 C (Fig. 8). Also, a rapid decrease in optical density at 320 nm was observed upon addition of trypsin to purified preparations of PVY and TEV-AV during the first five minutes of treatment. This rapid conversion was followed by a more gradual decrease in optical density to approximately 28 and 54%, respectively, of the original value after 30 min of treatment. This decrease in optical density continued for TEV-AV until the last determination two and a half hours later (Table VIII). Presumably, this is due to the disruption of some virus particles as observed for other plant viruses (Chidlow and Tremaine, 1971). Changes in optical density might result from the disruption of virus particles.

Aliquots taken from the spectrophotometer cells immediately after determining the decrease in optical density for each treatment, reacted serologically with their respective PVY or TEV antisera up to the 24 h treatment (Fig. 9). Some loss of antigenic specificity, however, was observed for TEV-AV coat protein following 24 h of treatment with trypsin whereas no change in specificity was noted for PVY even after 40 h exposure to trypsin (Fig. 9). Electron microscopic examinations of these PVY and TEV-AV preparations did not reveal any apparent change in the structural organization of the trypsin-treated virus particles.

Polyacrylamide gel electrophoresis of an active helper component preparation in the presence of SDS. At least 10 protein staining bands

Table VIII. Decrease in optical density at 320 nm of purified potato virus Y and tobacco etch virus upon treatment with trypsin for varying periods of time.

Length of Treatment (min)	Virus	
	PVY O.D.	TEV-AV O.D.
0	0.358	0.344
5	0.150	0.249
10	0.144	0.205
15	0.110	0.195
30	0.101	0.186
60	0.101	0.180
150	0.100	0.170

Figure 8. Polyacrylamide gel electrophoresis of the trypsin-treated coat protein subunits of purified potato virus Y (PVY) and tobacco etch virus (TEV-AV) in the presence of SDS. Both PVY (A) and TEV-AV (B) gels were prepared to a 6% polyacrylamide concentration. Samples from left to right are (a) untreated purified virus, (b) 5 min, (c) 10 min, (d) 15 min, (e) 30 min, (f) 60 min, and (g) 12 h treatments of purified virus incubated with trypsin for these periods of time.

a
b
c
d
e
f
g
A

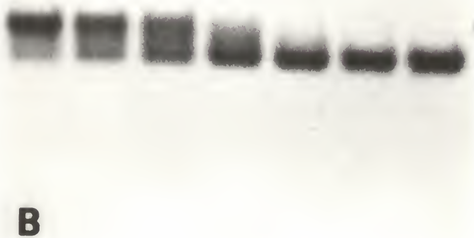
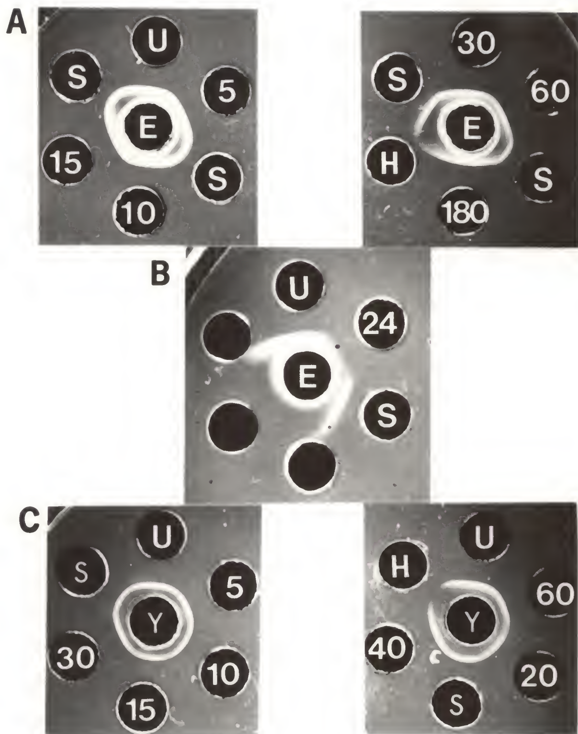

B

Figure 9. Double immunodiffusion tests with trypsin-treated potato virus Y and tobacco etch virus (TEV-AV) in a medium containing 0.8% Noble agar, 0.5% SDS, and 1.0% NaN_3 prepared in water. Center wells contain: (E) TEV antiserum, (Y) PVY antiserum. Peripheral wells contain: A) untreated freshly purified TEV-AV (U), TEV-AV treated with trypsin for 5, 10, 15, 30, 60, and 180 minutes, TEV-AV in sap extracted from infected 'Havana 425' tobacco (S), and healthy tobacco sap (H); B) partial loss of antigenicity of TEV-AV treated with trypsin for 24 hours (24), (U) and (S) as above; C) untreated freshly purified PVY (U), PVY treated with trypsin for 5, 10, 30, 60 minutes, 20 and 40 hours, PVY in sap extracted from infected 'Havana 425' tobacco (S), and sap from noninoculated tobacco (H).

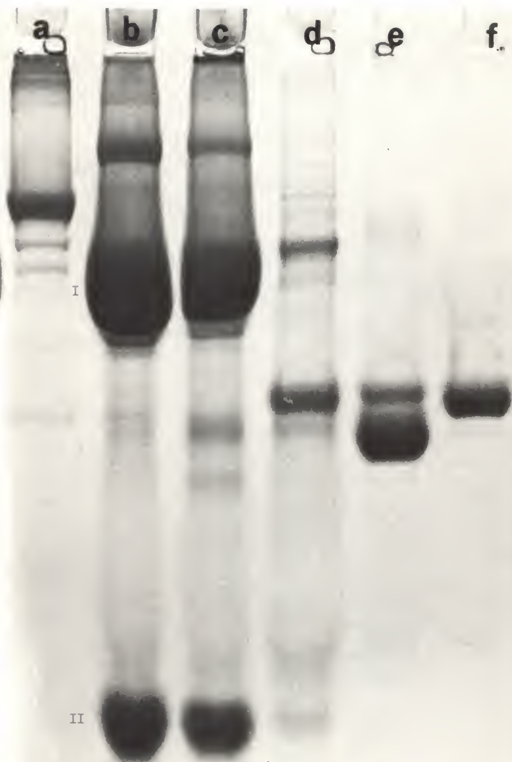

A
U
S
5
E
15
S
10
30
S
60
E
H
S
180
B
U
24
E
S
C
U
S
5
Y
30
10
15
U
H
60
Y
40
20
S

were resolved upon electrophoresis of an active helper component preparation in a 10% acrylamide gel containing SDS (Fig. 10). The presence of protein bands in the position, where PVY coat protein comigrated in adjacent wells, was observed in both the helper and control preparations. The mediocre resolution achieved in these gels, however, did not permit a better discrimination of the proteins present in these preparations.

Molecular weight estimates of the protein components resolved in 10% polyacrylamide gels of helper component preparations ranged from 11,500 to 90,000 d (Fig. 11). One additional protein of molecular weight of about 100,000 d was present in both the helper and control preparations in a 6% gel. The two predominant protein bands (I and II) present in both preparations (Fig. 10) are probably the two constituent subunits of fraction I protein, ribulose diphosphate carboxylase (Kung, 1976). Overloading of gel samples was necessary in order to resolve all proteins present.

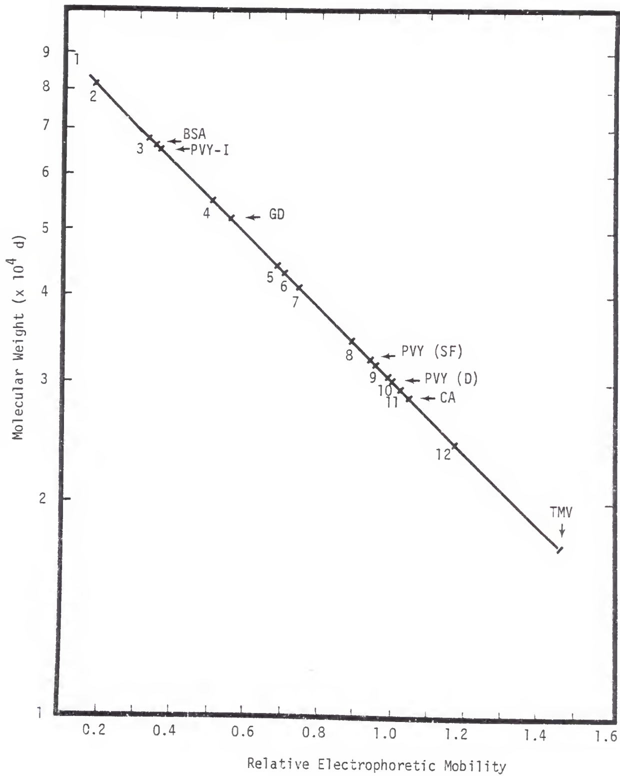
Polyacrylamide gel gradient electrophoresis. A better resolution of the proteins present in helper component and control preparations was achieved by polyacrylamide gradient electrophoresis in 12-16.5% thin gels. With this system, at least 30 proteins were resolved for the above preparations (Fig. 12). Overloading of protein samples, again, was necessary for resolution of the proteins present in these preparations. Protein staining bands were observed in helper component preparations at the position to which PVY coat protein migrated in adjacent wells.

Figure 10. Electrophoresis of potato Y virus, inclusion, and helper component preparations in a 10% polyacrylamide gel containing SDS. Samples from left to right are (a) PVY inclusion subunits, (b) control for helper component obtained from noninoculated plants, (c) PVY helper component preparation, (d) partially purified PVY, (e) purified PVY stored at 4 C for one year, (f) freshly purified PVY.



a
b
c
d
e
f
I
II

Figure 11. Comparison of the molecular weights of the proteins resolved in a 10% polyacrylamide gel upon electrophoresis of SDS-dissociated potato Y virus, inclusion, and helper component preparations. Molecular weight estimates for these proteins are (1) over 90,000 d, (2) 82,000 d, (3) 69,000 d, (4-5) 57-45,000 d, (6) 44,000 d, (7) 42,500 d, (8) 35,200 d, (9) 33,000 d, (10) 31,700 d, (11) 29,500 d, (12) 25,000 d, (13) 11,500 d. Potato virus Y MW estimates are inclusion subunit (PVY-I), 66,500 d; coat protein subunit, in the slow form (PVY-SF), 33,500 d, in degraded form (PVY-D), 30,500 d. Protein markers are (BSA) bovine serum albumin, 67,000 d; (GD) glutamate dehydrogenase, 53,000 d; (CA) carbonic anhydrase, 29,000 d; (TMV) tobacco mosaic virus coat protein subunit, 17,500 d.



Molecular Weight (x 10^4 d)
Relative Electrophoretic Mobility
9
8
7
6
5
4
3
2
1
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1
2
3
4
5
6
7
8
9
10
11
12
BSA
PVY-I
GD
PVY (SF)
PVY (D)
CA
TMV

Figure 12. Electrophoresis of a purified PVY-helper component preparation in a polyacrylamide gradient gel. Samples from left to right are (a) PVY coat protein subunit, (b) control preparation obtained from noninoculated 'Havana 425' tobacco, (c) PVY-helper component preparation, (d and e) same as b and c, respectively, but samples diluted two-fold. Arrow indicates approximate position of PVY coat protein subunits in the gel.

a
b
c
d
e

Cellulose Acetate Electrophoresis

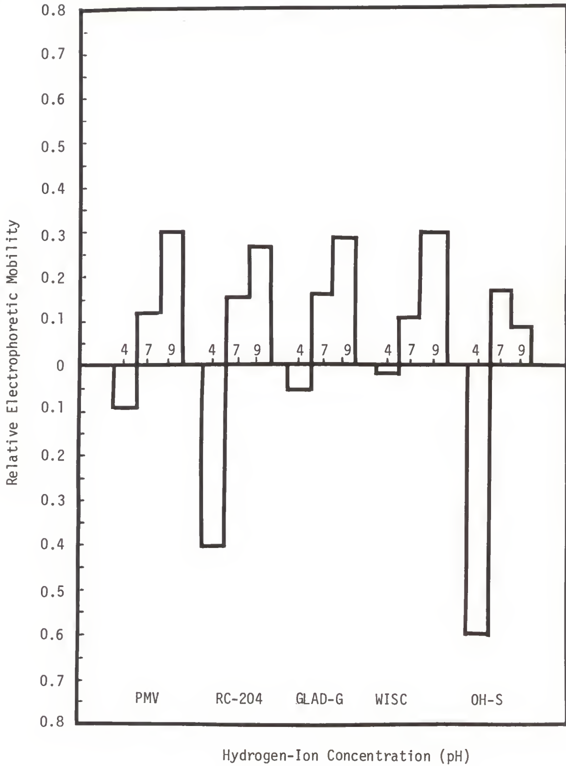
Bean yellow mosaic virus isolates. Electrophoresis of purified BYMV isolates on cellulose acetate plates revealed considerable differences in their electrophoretic mobility at the three pH values tested (Fig. 13). As expected for potyviruses with isoelectric points between 4.5-5.5 (Purcifull, 1966), all five of the BYMV isolates migrated towards the anode at pH 7.0 and 9.0, and towards the cathode at pH 4.0.

Considering these isolates in a decreasing magnitude of aphid transmissibility (OH-S, RC-204, PMV, GLAD-G, and WISC) (Table III), there was a direct relationship between aphid transmissibility and electrophoretic mobility in the cationic system (pH 4.0) and an inverse relation in the anionic system at pH 9.0 (Table IX). It was not possible to draw any conclusion from the results obtained at pH 7.0 due to the diverse electrophoretic behavior of the viruses in this system.

Tobacco etch virus isolates. A similar degree of variability in the electrophoretic mobility of the four TEV isolates was observed in these tests (Fig. 14). However, since the TEV-H and TEV-AV isolates were relatively more readily transmitted by aphids than TEV-AT, and TEV-NAT was not transmitted, the relationship between aphid transmissibility and electrophoretic mobility seems to be direct at pH 9.0 and to a lesser degree at pH 7.0, and inverse at pH 4.0 (Table X).

Potato Y virus and inclusions. Purified PVY was determined to have a higher electrophoretic mobility at pH 4.0 than at either pH 9.0 or 7.0. Purified PVY-inclusions did not migrate in these tests at either pH 9.0 or 7.0 towards the anode.

Figure 13. Cellulose acetate electrophoresis of five isolates of bean yellow mosaic virus at three hydrogen-ion concentrations. Viruses migrated towards the cathode at pH 4.0, and towards the anode at pH 7.0 and 9.0.



0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Relative Electrophoretic Mobility
4 7 9
4 7 9
4 7 9
4 7 9
4 7 9
PMV
RC-204
GLAD-G
WISC
OH-S
Hydrogen-Ion Concentration (pH)

Table IX. Relative electrophoretic mobility of bean yellow mosaic virus isolates through cellulose acetate at three hydrogen-ion concentrations.

Virus Isolate	pH of Buffer System[a]		
	4.0	7.0	9.0
OH-S	0.59[b]	0.16	0.07
RC-204	0.39	0.13	0.26
PMV	0.09	0.11	0.29
GLAD-G	0.05	0.14	0.28
WISC	0.01	0.10	0.35

[a] pH 4.0:potassium acetate-Beta-alanine acetic ac.; pH 7.0:sodium phosphate; pH 9.0:Tris-borate-sulfate.

[b] Distance migrated by protein over distance migrated by tracking dye.

Figure 14. Cellulose acetate electrophoresis of potato Y virus and inclusions, four isolates of tobacco etch virus, and tobacco mosaic virus at three hydrogen-ion concentrations. Viruses migrated towards the cathode at pH 4.0, and towards the anode at pH 7.0 and 9.0. Potato virus Y inclusions migrated in these tests only at pH 4.0, towards the cathode.

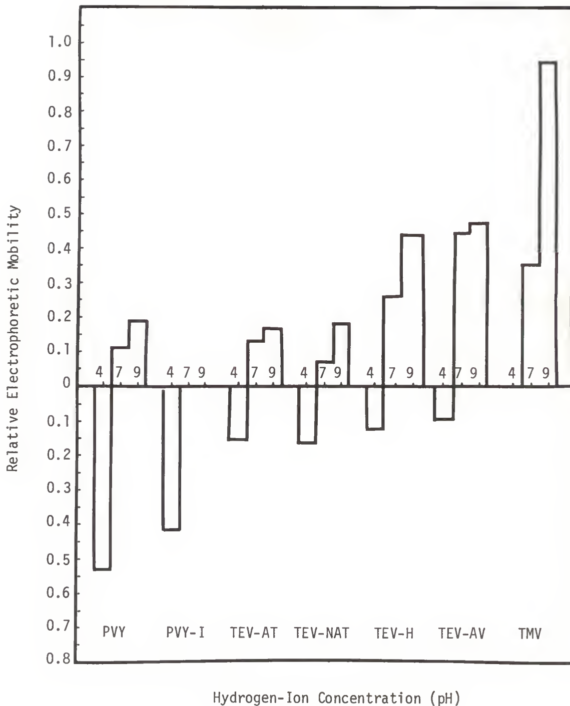

Relative Electrophoretic Mobility
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
4 7 9
4 7 9
4 7 9
4 7 9
4 7 9
4 7 9
4 7 9
PVY
PVY-I
TEV-AT
TEV-NAT
TEV-H
TEV-AV
TMV
Hydrogen-Ion Concentration (pH)

Table X. Relative electrophoretic mobility through cellulose acetate of purified potato virus Y coat and inclusion protein and tobacco etch virus coat protein at three hydrogen-ion concentrations.

Virus Isolate	pH of Buffer System[a]		
	4.0	7.0	9.0
PVY	0.53[b]	0.11	0.20
PVY-I	0.42	0.00	0.00
TEV-AT	0.16	0.14	0.18
TEV-NAT	0.17	0.06	0.19
TEV-H	0.12	0.27	0.45
TEV-AV	0.10	0.45	0.47
TMV[c]	0.00	0.36	0.94

[a] pH 4.0:potassium acetate-Beta-alanine-acetic ac.: pH 7.0:sodium phosphate; pH 9.0:Tris borate-sulfate.

[b] Distance migrated by protein over distance migrated by tracking dye.

[c] TMV = tobacco mosaic virus was included as control.

Tobacco mosaic virus. Purified tobacco mosaic virus showed considerable electronegativity when assayed at pH 9.0 and intermediate mobility at pH 7.0. At pH 4.0, tobacco mosaic virus did not migrate towards the cathode.

Cellulose acetate electrophoresis of the stored viral coat proteins of six selected potyviruses. Since in preliminary experiments the relative electrophoretic mobility of purified potyviruses seemed dependent upon time of storage before electrophoresis on cellulose acetate, several virus preparations maintained at 4 C for varying periods of time were compared with freshly purified preparations by the above technique. The results from these tests (Table XI) demonstrated that potyviruses with high electrophoretic mobilities at pH 4.0 (PVY, OH-S, RC-204) show a decrease in mobility at this pH upon storage, while the reverse is true at pH 9.0. Those viruses having an intermediate or low electrophoretic mobility at pH 4.0 (PMV, TEV-AT, TEV-NAT) did not show the same effect, migrating comparatively faster at all pH's tested. A parallel experiment with the PMV isolate of BYMV suggests that the electrophoretic behavior of this potyvirus is altered upon degradation of its coat protein (Fig. 15). Sometimes two protein species were observed on cellulose acetate strips after electrophoresis at pH 9.0 of PMV preparations containing the two molecular weight components resolved by SDS-PAGE (Fig. 15b). The slower migrating protein species (not shown) had approximately the same relative electrophoretic mobility of the only species resolved at pH 9.0 with freshly purified PMV.

Table XI. Cellulose acetate electrophoresis of six potyviruses stored for varying periods of time after purification.

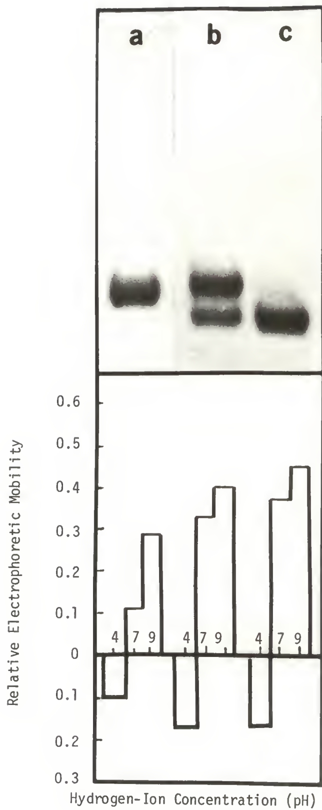
Virus	Preparation	pH of Buffer System[a]		
		4.0	7.0	9.0
PMV	F	0.09[b]	0.11	0.29
	IS	0.16	0.33	0.40
	S	0.16	0.37	0.45
RC-204	F	0.39	0.13	0.26
	S	0.16	0.33	0.67
OH-S	F	0.59	0.16	0.07
	S	0.40	0.04	0.30
TEV-AT	F	0.16	0.14	0.18
	S	0.20	0.39	0.40
TEV-NAT	F	0.17	0.06	0.19
	S	0.39	0.17	0.38
PVY	F	0.53	0.11	0.20
	S	0.48	0.24	0.31

[a] pH 4.0:potassium acetate-Beta-alanine-acetic ac.; pH 7.0:sodium phosphate; pH 9.0:Tris borate-sulfate.

[b] Distance migrated by protein over distance migrated by tracking dye.

[c] F = freshly purified virus; IS = stored for 15 days at 4 C after purification; S = stored longer than a month.

Figure 15. Effect of capsid protein heterogeneity on the electrophoretic mobility of the pea mosaic isolate of bean yellow mosaic virus at three hydrogen-ion concentrations. Samples from left to right are (a) undegraded, (b) partially degraded, and (c) degraded virus assayed by SDS-PAGE (top figure), and cellulose acetate electrophoresis (bottom figure). Migration of viruses through cellulose acetate was towards the cathode at pH 4.0 and towards the anode at pH 7.0 and 9.0.


a
b
c
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
4 7 9
4 7 9
4 7 9
Relative Electrophoretic Mobility
Hydrogen-Ion Concentration (pH)

DISCUSSION

This study reinvestigated the evidence presented by Govier, Kassanis, and Pirone (1977) indicating that a protein component other than the viral coat or inclusion protein is responsible for the dependent aphid transmission phenomenon of potyviruses, and evaluated the electrostatic properties of the coat protein of several potyviruses in relation to their aphid transmissibility.

This investigation confirmed the results obtained in previous works (Kassanis and Govier, 1971a, b; Simons, 1976; and Paguio and Kuhn, 1976) which demonstrated the aphid transmissibility of normally nonaphid-transmissible plant viruses in the presence of certain potyviruses referred to as helpers. The helper activity of potato virus Y (PVY) was also demonstrated herein with a vectorless isolate of tobacco etch virus. However, only one of seven potyviruses tested for helper activity in this study aided the aphid transmission of a nonaphid-transmissible isolate of bean yellow mosaic virus. Previous attempts to effect dependent transmission of vectorless isolates of this virus had been unsuccessful (Kamm, 1969; Evans and Zettler, 1970).

The three isolates of dasheen mosaic virus (DMV) tested exhibited an equivalent degree of transmissibility by the aphid _Myzus persicae_. The differential transmissibility of the Florida isolate (DMV-FL) by _M. persicae_ and _Aphis craccivora_ and the inability of _Pentalonia nigronervosa_ to transmit this virus, constitute further evidence of the

phenomenon of vector specificity as described by other workers (Pirone, 1969). The possibility of increasing the aphid transmissibility of DMV by allowing aphids to probe first on plants infected with other potyviruses seems remote based on the results obtained in this trial. It was demonstrated, however, that up to 90% transmission of DMV-FL can be achieved under laboratory conditions by simply increasing the number of aphids (20) used to inoculate each test plant. Attempts to correlate these results with the electrophoretic properties of DMV, however, were prevented by the inability to obtain sufficient quantities of the three isolates in purified form for cellulose acetate electrophoresis.

It has been demonstrated that the phenomenon of dependent transmission provides a mechanism of dissemination for some vectorless plant viruses. This observation provided the basis for a control measure for potato aucuba mosaic virus in Great Britain through certification programs that guarantee freedom of its helper viruses from commercial planting stock (Kassanis, 1961). The dependence on a helper virus also seems to be the factor responsible for the limited dissemination of a vectorless strain of peanut mottle virus in the field (Paguio and Kuhn, 1973).

The results obtained in this study confirmed the report by Govier and Kassanis (1974a, b) that supernatants prepared by ultracentrifugation of extracts obtained from PVY-infected plants contain a helper component for aphid transmission of purified PVY from artificial membranes. Assays of an active helper component preparation by SDS-PAGE, however, failed to reveal the presence of the virus-induced protein of molecular weight 100,000-200,000 d described by these workers. The results of this study were also in disagreement with those reported by Govier _et al_.

(1977) in that protein bands in the zone where PVY coat protein migrated in adjacent wells were observed in polyacrylamide gels of both the helper component and control preparations. Furthermore, these assays revealed the presence of at least 30 additional proteins in both preparations. This observation could account for the failure of Govier et al. (1977) to produce a specific antiserum to the helper component prepared according to their purification procedure. Attempts to demonstrate the presence of PVY coat or inclusion protein in an active helper component preparation using several specific antisera were unsuccessful in this study. It is therefore unlikely that the helper component is made up of capsid or inclusion protein subunits, since in the case of PVY, dissociated virus and inclusion protein subunits are known to react with their respective antisera prepared against undissociated virus particles or inclusions (Purcifull and Batchelor, 1977).

An alternative possibility is that a portion rather than the entire capsid protein subunit is the helper component. The experiments conducted in this study with the pea mosaic isolate of BYMV indicated that neither the temperature regimes, nor the ageing periods selected, appreciably affected the capsid protein of this virus. However, nine potyviruses assayed by SDS-PAGE revealed capsid protein heterogeneity and further degradation of their coat proteins upon storage of purified preparations at 4 C. This process resulted in a loss in molecular weight of about 5,000 d as demonstrated previously for other potyviruses (Hiebert and McDonald, 1973, 1976). The conversion of the slow into the fast form of the viral coat protein of PVY and TEV was also achieved in this study upon incubation of these potyviruses with trypsin. This constitutes the first demonstration of the degradation of the viral coat

protein of a potyvirus by selective enzymatic cleavage. It appears from these studies that the critical factor in avoiding degradation is the separation of the virus from the bulk of contaminant host proteins early in the purification procedure. The use of differential centrifugation as described by Govier and Kassanis (1974b) for the purification of PVY, seems to be conducive to a higher degree of coat protein degradation because of the initial concentration of the virus and most host cell components by ultracentrifugation.

This study also provides evidence indicating that the loss of the labile portion, upon conversion of the slow into the fast form of the viral coat protein of trypsin-treated PVY and TEV did not result in a loss in serological specificity. Further degradation of the viral coat protein beyond the conversion to the fast form, however, can result in some loss in antigenic specificity as observed in this study for trypsin-treated TEV. These results, however, must be viewed with caution since the demonstration of serological differences between the degraded and undegraded capsid proteins of potyviruses is dependent upon the specificity of the antiserum used.

The cellulose acetate electrophoresis assay of the 10 potyviruses selected for these experiments revealed considerable variability in the electrophoretic properties of these viruses at the three pH's tested. The electrophoretic mobility of some of these potyviruses tested after incubation of purified preparations at 4 C for varying periods of time was further modified. An experiment with the pea mosaic isolate of BYMV revealed that the altered electrophoretic mobility through cellulose acetate of stored purified preparations occurred simultaneously with the conversion of the slow to the fast form of the capsid protein of this virus as determined by SDS-PAGE.

Since cellulose acetate, unlike polyacrylamide gels, has a negligible sieving effect and thus allows proteins to migrate according to their net charge, the results obtained in this study suggest that the anomalous electrophoretic behavior of the degraded coat protein of potyviruses is due to the loss of charged aminoacids present in the labile portion. The increased electronegativity at pH 9.0 shown by the stored potyviruses tested by cellulose acetate electrophoresis, and the high sensitivity of the coat protein to trypsin, which selectively attacks positively charged aminoacids (arginine and lysine), provide support to the above suggestion.

Since most cell membranes in animal or plant cells possess a net negative charge (Tolmach, 1957), the presence of basic or positively charged aminoacids on the capsid protein of potyviruses could be required for attachment of virus particles to receptor surfaces on their aphid vectors' mouthparts. Release and transmission of the virus would then be brought about by a change in pH or ionic strength induced by the ingestion of sap or by salivary secretions. Following this hypothesis, a direct correlation between aphid transmissibility and electrophoretic mobility at pH 4.0 (potyviruses migrated towards the cathode at this pH) was observed for the BYMV isolates tested. The appreciable electronegativity shown by PVY at this pH would also be in accordance with its superior helper activity. Conversely, the lack of electrophoretic mobility of tobacco mosaic virus (TMV) at pH 4.0, could be taken as an indication of its inability to be transmitted by aphids. This hypothesis, however, could not be substantiated by the results obtained in the trials with the four isolates of TEV, since the two isolates that were more readily transmitted (TEV-H and TEV-AV) exhibited a higher

electronegativity at pH 9.0 and a lower electropositivity at pH 4.0 than the other two isolates which had a relatively low (TEV-AT) or no (TEV-NAT) aphid transmissibility. It is possible, however, that TEV behaves anomalously due to the high content of acidic aminoacids in its capsid protein (Damirdagh and Shepherd, 1970) so that the loss of basic (positively charged) aminoacids is offset by a concomitant loss of acidic (negatively charged) aminoacids with the labile portion. There is also the possibility that although cell membranes carry a net negative charge, both positive and negative charges are involved in the electrostatic attachment of virus particles to cell receptor sites.

A similar hypothesis has been proposed for the nematode transmitted viruses by Harrison, Robertson, and Taylor (1974). According to these authors, the adsorption of these viruses to the inner surface of the guide sheath or esophagous of their nematode vectors would be determined by the surface charge of virus particles. In a later publication, Taylor and Robertson (1977) elaborate on the same hypothesis, according to which, the virus must have a net positive charge in order to adsorb to negatively charged surfaces on the nematode's receptor sites. No experimental evidence, however, was presented to support their hypothesis. It is worth mentioning in this respect, that as in the case of nematode-transmitted viruses, aphid-borne potyviruses have not been observed inside the cells of their vectors but only adsorbed to specific sites on their mouthparts (Taylor and Robertson, 1974; Lim et al., 1977). In the study by Lim et al. (1977), little virus was observed to be adsorbed to the mouthparts of an inefficient aphid vector. Vector specificity in this case would be determined by the ability of a particular aphid species to adsorb virus particles.

The exact molecular weight of the helper component has not been determined. The 100,000-200,000 d estimate given by Govier et al. (1977) was obtained from gel filtration and ultrafiltration studies using materials with wide fractionation ranges. As recognized by the authors themselves, these methods often yield erroneous estimates due to their inability to detect aggregation of the proteins being assayed. Potato virus Y, which has a genome consisting of single stranded RNA with a molecular weight of about 3.2×10^6 d (Hinostroza-Orihuela, 1975), is presumed to have the genetic capability of coding for proteins with a combined weight of approximately 320,000 d. Two of the proteins consistently associated with the infection process of potyviruses are the viral capsid and inclusion proteins. In this study, the total molecular weight of the capsid protein and inclusion protein subunits was calculated to be 100,700 d. To this value, one must add the weight of the viral replicase which for a smaller virus with helical structure, such as tobacco mosaic virus (TMV), is approximately 130,000 d (Zaitlin et al., 1973). It is not known whether this replicase is made up of subunits, however. Nevertheless, it is still theoretically possible for a protein of about 100,000-200,000 d to be coded as an aphid transmission factor. Evidence for the translation of such a protein, however, was not obtained by Siegel and Hari (1977) in their work on the translation of the RNA genomes of PVY and TEV in tobacco tissue. Translation of PVY m-RNA resulted in the demonstration of four virus-induced or virus-stimulated proteins with molecular weights of 65, 50, 41, and 32×10^3 d. The 65,000 and 32,000 d proteins could correspond to the viral inclusion and capsid subunits, respectively. These authors also found a low molecular weight RNA component (approximately 350,000 d) in extracts

of PVY or TEV infected tissue. A similar component described for TMV (Hunter et al., 1976) was shown to be an efficient monocistronic messenger for capsid protein. The function of the low molecular weight component of PVY and TEV was not determined by Siegel and Hari (1977). However, considering the molecular weight of the PVY coat protein subunit (33,700 d), it is likely that this RNA component had the same function as that of TMV and coded for capsid protein.

The evidence presented in this study with several potyviruses, indicates that proteolytic enzymes catalyze the cleavage of the labile portion of the capsid protein which contains, among others, charged aminoacids. The loss of these aminoacids is apparently responsible for the observed modification of the electrophoretic properties of potyviruses. The demonstration that the labile portion of the viral coat protein is involved in the aphid transmission phenomenon is complicated by the inability of aphids to transmit potyviruses in purified form. This inability to transmit purified potyviruses (Pirone and Megahed, 1966; Govier and Kassanis, 1974a, b), however, could simply reflect the failure to provide these viruses with adequate pH, ionic strength, or temperature conditions so that they can be adsorbed to their vectors' mouthparts. Considerable work is needed on this area. Extraction of potyviruses from infected plants in the presence of proteolytic-enzyme inhibitors could also be studied. The success or failure to transmit degraded potyviruses in the presence of the helper component would be equally influenced by its sensitivity to proteolytic enzymes (Govier et al., 1977), or by the effect of the subsequent treatments to remove or neutralize these enzymes on the virus. Transmission of degraded potyviruses in the presence of the helper component, on the other hand,

would not provide any significant information on the nature of the helper component itself. The demonstration of the origin of the protein responsible for the dependent transmission phenomenon of potyviruses necessitates a direct approach.

Perhaps the most promising technique that can be used initially to further purify and characterize the helper component is that of affinity chromatography. This technique would involve the production of a specific antiserum to the helper component. If the purification procedure could not be improved without loss of biological activity, the antiserum could be absorbed with a control preparation obtained from noninoculated plants, and then fractionated. The antibodies in the specific fraction can then be covalently adsorbed to a gel matrix in a column where they would act as ligands for their homologous antigen. Desorption of the helper component protein would then be accomplished by altering the pH and/or slat concentration of the eluant. Due to the lability of this protein, this work must be conducted at 0-4 C, and a proper biological assay performed with the recovered fractions to demonstrate helper activity. Once its purity, molecular weight, and aminoacid composition and terminal sequence were known, it should be possible to determine whether the helper component is an integral part of the viral capsid or a previously unrecognized protein coded for by aphid transmissible potyviruses in infected plants.

It is the contention of this study that the lability of the capsid protein and its effect on the electrostatic behavior of potyviruses has not been taken into account in the characterization studies of the helper component. The determining role of the viral coat protein in the dependent aphid transmission of barley yellow dwarf virus was

demonstrated by Rochow (1970). Although this virus has a persistent mechanism of transmission, the possible role of the viral capsid in the nonpersistent transmission phenomenon of potyviruses can not be ruled out.

This study is the first experimental investigation of the possible role of the electrostatic properties of the viral capsid protein in the aphid transmission phenomenon of plant viruses.

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BIOGRAPHICAL SKETCH

Francisco Jose Morales Garzon was born in Cali, Colombia, on February 20, 1948. He received his primary and secondary education at the San Bartolome la Merced and Emmanuel d'Alzon Schools, Bogotá. In 1971, he obtained a Bachelor of Science degree in agronomy and agronomic engineering from the National University of Colombia at Bogotá. In the same year, he traveled to the United States where he entered the Graduate School of Cornell University with a major in plant pathology and a minor in international agricultural development. He was awarded the Master of Science degree in June, 1974. In September, 1974, he entered the University of Florida to commence work towards a Doctor of Philosophy degree in plant pathology.

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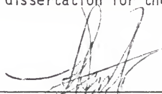
He married the former Lea Dobrzynski of Colombia on October 28, 1972.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Dr. F.W. Zettler, Chairman
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dr. J.R. Edwardson
Professor of Agronomy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

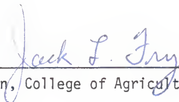
Dr. D.R. Pring
(ARS-USDA) Associate Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dr. E. Hiebert
Associate Professor of Plant Pathology

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June 1978



Dean, College of Agriculture

Dean, Graduate School